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Non-Major Histocompatibility Complex-Associated Genetic  
Resistance to Marek's Disease: Expression at the Target Cell  
Level

by



William Michael Gallatin

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF DOCTOR OF PHILOSOPHY

IN

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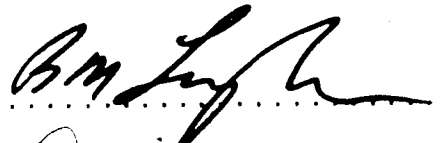
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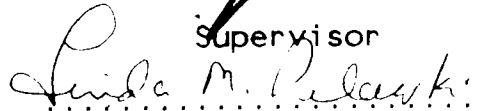


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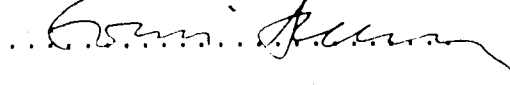


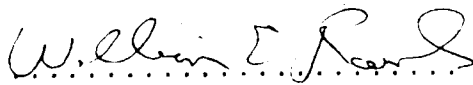
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## ABSTRACT

The mechanisms responsible for non-major histocompatibility complex (MHC)-associated genetic resistance to Marek's disease (MD) - were studied in resistant line 6 and susceptible line 7 chickens. Line-specific differences, in the capacity of lymphocytes, but not fibroblasts, from these lines to adsorb and replicate the herpesvirus of turkeys (HVT) in vitro were demonstrated. Spleen cells from SPF, adult line 6 and line 7 birds were exposed to MDV in vivo in irradiated histocompatible, third-party recipients. A higher frequency of MDV infected cells was observed in spleen and peripheral blood of the recipients of line 7 cells than in birds which received line 6 cells. Since there was evidence that the lymphocytes of the susceptible and resistant lines responded differently to herpesvirus infection in vitro, attempts were made to increase MD susceptibility in resistant birds by adoptively transferring embryonic line 7 spleen cells into day 15 line 6 embryos. No significant increase in MD mortality was observed. Transplantation of either normal or gamma-irradiated (1000r) line 7 thymuses into line 6 hatchlings significantly increased the incidence of MD lymphomas. Similar increases in MD mortality were achieved by adoptive transfer of adult line 7 spleen cells into line 6 recipients. No protective effect was observed following transfer of spleen cells from line 6 donors into susceptible line 7 recipients. To determine if the thymic

microenvironment influences the target cell phenotype, line 6 and line 7 thymuses were irradiated in situ and then grafted to the chorioallantoic membranes of line 6 resistant embryos. Line 6 stem cells which repopulated line 7 grafts, were found to have a higher HVT adsorption capacity than those cells which repopulated the syngeneic line 6 grafts. Thymic stromal cells from these lines did not differ in their ability to adsorb infectious HVT. Monoclonal antibodies against HVT were produced. Of the nine antibodies which were positive for anti-HVT activity in initial ELISA and agglutination tests, two were selected for further characterization. These reagents, HVT.1 and HVT.2, were of the IgG class as determined by comparison of their Sephacryl-300 elution profiles with those of known IgG and IgM monoclonal antibodies. Both HVT.1 and HVT.2 neutralized infectious HVT in vitro. The antigenic specificity they detected was also present on the surface of infected chick embryo fibroblasts (CEF) as demonstrated by the binding of HVT.1 and HVT.2 coupled, fluorescinated, latex beads to HVT infected but not uninfected CEF. HVT.2 was used in the in vitro antibody directed rosette assay (ADRA) to determine the frequency of virus binding cells in the lymphoid organs of line 6 and line 7. Virus binding leukocytes were more frequent in the susceptible line.

The evidence presented here is supportive of the notion that genetic resistance to MD in line 6 and 7 is manifested at the level of a direct interaction between MDV and its

target T-lymphocyte. Further, the thymic microenvironment may play a key role in this event.

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## List of Abbreviations

ADCC -	Antibody-dependent cellular cytotoxicity
ADRA -	Antibody-directed rosette assay
ALL -	Avian lymphoid leukosis
BL -	Burkitt's lymphoma
CAM -	Chorioallantoic membrane
CEF -	Chick embryo fibroblasts
CKC -	Chick kidney cells
CMI -	Cell-mediated immunity
CRBC -	Chicken red blood cell
EA -	Early antigens
EBV -	Epstein-Barr virus
ELISA -	Enzyme-linked immunosorbent assay
FBS -	Fetal bovine serum
FFU -	Focus forming units
FIA -	Focus inhibition assay
GVHR -	Graft versus host reaction
HSV-2 -	Herpes simplex virus type 2

HVA -	<u>Herpesvirus ateles</u>
HVS -	<u>Herpesvirus saimiri</u>
HVT -	Herpesvirus of turkeys
HVT.1 -	monoclonal antibody to HVT
HVT.2 -	monoclonal antibody to HVT
IM -	Infectious mononucleosis
MA -	Membrane antigen
MATSA -	Marek's disease tumour associated surface antigen
MD -	Marek's disease
MDV -	Marek's disease virus
MHC -	Major histocompatibility complex
MULV -	Murine leukemia virus
NK cells -	Natural killer cells
NPC -	Nasopharyngeal carcinoma
SPF -	Specific pathogen free
VCA -	Virus capsid antigen
VMA -	Virus membrane antigen
VN antibodies -	Virus neutralizing antibodies

## I. LITERATURE REVIEW

### A. Marek's Disease: Historical Aspects

#### Introduction

Although Josef Marek first described the paralytic syndrome as a polyneuritis of the domestic fowl (Gallus domesticus, Marek, 1907), more than half a century was to pass before the lymphoproliferative syndrome we know today as Marek's disease (MD) came to bear his name. During the intervening years a number of different names have been applied to the disease. These include polyneuritis, neurolymphomatosis gallinarum, fowl paralysis, range paralysis, big liver disease, iritis, visceral lymphomatosis, avian leukosis complex, type II avian leukosis and acute avian leukosis (Payne et al., 1976; Calnek, 1980). The first section of this review of the literature will be concerned primarily with the major historical points in the evolution of Marek's disease as a well defined clinical entity with a known aetiological agent, Marek's disease herpesvirus (MDV). The more intricate details of the natural history of MDV infection in the domestic chicken and host factors which determine the outcome of infection will be dealt with in the chapters which follow. Other general reviews are available on the subject of MD (Biggs, 1968, 1972; Payne, 1972, 1980; Nazerian, 1973). The most comprehensive review is that of Payne and his associates written in 1976. Witter's 1972

paper is perhaps the best summary of the epidemiological aspects of MD while the more recent, 1980 analyses by Nazerian and by Calnek provide respectively the best sources of information on the molecular biology of MDV replication and host immunity to MD.

Early Description of the Classical Form of Marek's Disease

The early descriptive papers written in the 1907-1960 period present a different picture of the histopathology of the disease now known as MD. While the most common and economically most important form evident today has as its major feature the development of frank, T-cell lymphomas of the visceral organs (Calnek, 1980), the disease reported by Marek in 1907 was of a very different sort. In his original paper he outlined a syndrome of paresis and paralysis which occurred naturally in domestic fowl and had as its principle histopathology an edematous infiltration of the peripheral nerves by mononuclear blood leukocytes. The incidence was less than 5% of the birds in a given infected flock. Marek viewed the degeneration of the nerves as being secondary to lymphoid infiltration and, thus, coined the name polyneuritis. Indeed, for many years thereafter, no neoplastic component was evident to those who studied the disease (Payne et al., 1976).

• The first really comprehensive study of polyneuritis or fowl paralysis as it had become known in the United States was reported by Pappenheimer and his colleagues in 1929 (Pappenheimer et al., 1929a, 1929b). In addition to the

lesions described by Marek which were found to occur in the peripheral but not the central nervous system, these workers reported a lymphomatous growth in the ovary which was invasive in character. These authors suggested a new name, neurolymphomatosis gallinarum, which included this more neoplastic characteristic. Intraabdominal inoculation of young chicks with homogenates of the nerve lesions induced a similar array of lesions in a small percentage of recipients. The agent responsible for transmission was not, however, able to pass through a micropore filter.

This latter property of the agent in question was a source of confusion for many years. The causative organism did not fit the description of the then known, filterable avian tumour viruses of which Rous sarcoma virus is an example (reviewed by Vogt, 1965). To further confound the problem, other avian leukoses which were caused by filterable viruses had been reported (Ellerman, 1921). Early attempts to distinguish between these RNA virus-induced lymphomas and the T-cell lymphomas sometimes seen in fowls were very difficult for scientists of the era (Patterson et al., 1932). It is not surprising then, that workers of this period, lacking our current knowledge of lymphomas and of electron microscopy and RNA and DNA viruses, lumped both types of tumours under the common heading of leukosis (Calnek, 1980). For this reason, papers of this era concerning transmission and host resistance are extremely difficult to interpret clearly.



since more than one clinical entity was usually present in the experiments.

A paper which is illustrative of this point is that of Hutt and Cole in 1947 which is generally taken as the first demonstration that genetic selection for MD resistance was possible. These workers started with an unselected breeder stock which showed a mortality of 14.9% to all forms of "lymphomatosis". By contact exposure of commercial flocks for 10 generations over a twelve year period they selected resistant and susceptible lines of chickens. At the end of these selections mortality due to lymphoma was 25% in the susceptible line S and 7.9% and 8.3% in the two resistant lines, C and K, respectively. With our current knowledge of the differences in organ distribution of MDV and RNA virus-induced lymphomas, it is evident from Hutt and Cole's histopathological descriptions that the majority of deaths were MDV-induced. The fact remains, nonetheless, that the exact contributions of MDV and RNA tumour viruses to the overall mortality reported is unknown.

#### The Appearance of the Acute Form of Marek's Disease

Visceral lymphomas were known to occur concomitantly with the nerve lesions of paralysis or classical MD as early as the 1920's (Pappenheimer et al., 1929a). These tumours were relatively rare though with the occasional ovarian tumour being the most common form. This classical paralytic type of MD generally affected considerably less than 10% of the birds in any given flock and usually did not appear

until after fifteen weeks of age (Witter, 1972). Around 1950 the development of the lymphomatous form of the disease became more common although, as mentioned previously, the inclusion of other RNA virus-induced neoplasms in veterinary records of the time confuses the issue. Basing his argument on histopathological grounds, Campbell suggested in 1956 that fowl paralysis and avian leukosis were distinct clinical entities. He viewed the tumours of the former disorder as lymphogranulomas and not as true neoplasms. By the late 1950's however, it became obvious that a distinctly more acute form of lymphomatous disease was spreading rapidly through poultry flocks along the eastern seaboard of the United States (Benton and Cover, 1957). By 1965 Great Britain was also affected (Biggs et al., 1965). Peripheral nerve lesions of classical MD were still observed, but the new syndrome differed from the former malady in a number of important characteristics: 1) death could occur as early as four weeks post-hatching and peaked around ten to twelve weeks of age, 2) outbreaks of the disease were explosive affecting 20 - 30% of the birds in a given flock with mortality sometimes approaching 60%, and 3) a high frequency of visceral lymphomas in the lungs, kidney, liver, spleen, heart, mesentery and especially the gonads was observed (Benton and Cover, 1957; Biggs, 1961; Biggs et al., 1965; Dunlop et al., 1965; Payne, 1972). The name visceral lymphomatosis was applied by Benton and Cover while Dunlop and colleagues preferred the term acute leukosis. The

nomenclature first applied by Biggs in 1961 and restated in 1965 has proved to be the most useful and is now in general usage. According to Biggs' classification, the avian leukosis complex was split into three components: 1) avian lymphoid leukosis (ALL), the RNA virus-induced lymphomas which are of B-lymphocyte origin (Peterson *et al.*, 1966); 2) classical Marek's disease, a term descriptive of the paralytic form of MD, and 3) acute Marek's disease, the primarily lymphomatous type of MD.

Acceptance of Biggs' terminology accomplished a great deal in that it removed the red herring of RNA virus-induced tumours from MD research. Other questions did remain though regarding classical and acute MD. For example, were the lesions observed simply the result of an autoimmune reaction of the host instigated by some unknown viral pathogen, the so-called extrinsic theory, or were they the outcome of true neoplastic transformation of lymphoid tissue, the intrinsic theory (Payne, 1972)? The major proponent of the extrinsic theory, Wight, described the formation of nerve lesions in three stages having the following characteristics: type I, extensive cellular infiltration by small lymphocytes, plasma cells and few lymphoblasts with no edema; type II, extensive edema with little cellular infiltration; and type III, extensive proliferation of lymphoblasts. (Wight, 1962). The temporal description of Payne and Biggs, major advocates of the intrinsic theory, was, interestingly enough, almost the exact reverse. Their three stages of progression were the

following: type A, characterized by proliferating lymphoblasts with demyelination of peripheral nerves; type B, noted for the presence of small and medium lymphocytes in the nerve sheaths with some edema; and type C, observed primarily in older, sub-clinical birds with only a light infiltration of the nerve sheaths by small lymphocytes and plasma cells. The former author viewed lymphoblastic proliferation as secondary to the host response to viral damage to nerve tissue. The latter authors considered neoplastic transformation and cellular proliferation to be primary to nerve destruction. Although there is now little doubt that the lymphomas of acute MD (which correspond to Wight's type III and Payne and Biggs' type A lesions) are true neoplasms with autonomous proliferative capability (Calnek, 1980; Nazerian, 1980), the controversy over the nature of classical MD lesions has yet to be settled. An autoimmune component is at least suggested by studies which show the presence of antibodies against myelin in paralyzed birds (Wight and Siller, 1965; Ringer and Alshtar, 1968; Lampert et al., 1977).

One must remember that the real impetus for research in the field from 1960-1970 was an economic one. By the early 1960's losses in the poultry industry due to the epidemic of acute MD amounted to over \$150 million annually in the U.S. alone. Adding to this the losses in Great Britain and Europe brought MD into focus as a disease of enormous financial impact. The task of prime importance in the 1960's was,

therefore, to determine the mode of transmission and, hopefully, in doing so, identify the aetiological agent. As will become apparent in the section which follows, this identification was not easily accomplished.

#### The Discovery of a Herpesvirus as the Aetiological Agent of Marek's Disease

As an infectious aetiology of MD became more and more likely with each new outbreak in the eastern United States, attempts were begun in earnest to elucidate the mechanism of its transmission. It is true that Pappenheimer and his colleagues as well as others (Pappenheimer et al., 1929b; reviewed by Payne et al., 1976) in the 1930's had successfully transmitted MD experimentally, but the efficiency and reproducibility of transmission were quite low. Efficient experimental reproduction of acute MD was first accomplished by Sevoian and co-workers who inoculated 5000 line S chicks with homogenates of ovarian tumours and observed lymphomas in almost 100% of the recipients two to three weeks later (Sevoian et al., 1962). These workers claimed that the agent responsible, which they designated the JM isolate, was effective in transmitting MD in 50% of cases after millipore filtration. Numerous workers have attempted to repeat this filtration experiment without success (reviewed by Nazerian, 1980; Calnek, 1980). It seems likely with the benefit of hindsight that Sevoian et al. had another virus, possibly of the ALL group, present in addition to MDV. The efficacy of transmission of both acute

and classical MD by tumour homogenates and whole blood from affected fowl was, nonetheless, corroborated by the later work of others (Biggs and Payne, 1963, 1967; Owen et al., 1966; Purchase and Biggs, 1967). Although the incidence of disease in recipients varied with their genotype and age at inoculation (older birds being less susceptible), the results from the various laboratories were in good overall agreement on the transmissibility of MD. Of course, a major criticism of these experiments was that the newly arising tumours might well have been transplants of the original. Owen et al. demonstrated conclusively that this was not the case. Injection of cockrels with the HPRS-20 ovarian tumour isolate bearing the female, ZW, chromosome marker resulted in de novo induction of lymphomas which were wholly male, ZZ, in karyotype.

These studies on experimental transmission with whole cells were a step in the right direction, but it was extremely unlikely that MD arose via transfer of blood or tumour tissue from one bird to another. Therefore, an alternate means for its spread in the natural situation had to be determined. Chicks hatched in isolation from the eggs of infected flocks showed no signs of disease and thus vertical transmission was ruled out (Solomon et al., 1970; Solomon and Witter, 1973). Demonstration that MD was spread by air-borne mechanism was first indicated by Sevoian et al. (1963). who housed uninoculated line S chicks in an isolator connected to the airflow outlet of another isolator

containing chicks experimentally infected with the JM isolate. Twenty-four of thirty such indirectly exposed birds died of acute MD while none of the isolated controls died. Other workers showed that following experimental injection of infected whole blood into day-old chicks there was a two-week incubation period before the infection could be passed on by contact exposure (Kenzy and Biggs, 1967). Neither of these groups established the identity of the aerosolized agent even though its existence was strongly suggested.

The major obstacle in the way of identification of the aetiological agent of MD was that no in vitro method for its propagation existed until 1967. In that year Churchill and Biggs reported that co-cultivation of tumour cells or whole blood from chickens carrying the HPRS-16 isolate of acute MD with chicken kidney cell (CKC) monolayers induced a focal cytopathic effect (CPE) in the CKC. The CPE was sensitive to inhibitors of DNA synthesis and could be passaged serially with trypsinized cells. On closer examination of the syncytial foci with the electron microscope numerous intracellular virus particles were observed. These had the morphological characteristics of herpesviruses. Finally, CKC from cultures with CPE but not those from control cultures were found to induce acute MD upon inoculation of young chicks. Identical results using a different MD isolate and duck embryo fibroblast indicator cells were obtained the following year by Witter and his associates (Witter et al.,

1968a; Solomon et al., 1968; Nazerian et al., 1968). In both cases the capacity of cultured cells to induce MD in vivo was strictly cell-associated, not filterable and correlated directly with the degree of the CPE in the cultures. On the basis of their findings both groups stated that the virus conformed to the properties of the B-subgroup of herpesviruses described by Melnick et al. (1963). They suggested that it was the aetiological agent of MD. In the same year, the presence of a B-group herpesvirus in CKC cultures showing a CPE was confirmed by additional electron microscope studies. Quite significantly though, in these experiments there was no evidence of virus particles in fresh tumour cells from which the CKC infection had, presumably, been derived (Ahmed and Schidlovsky, 1968; Nazerian and Burmester, 1968). Thus the infection in lymphoma cells appeared to be a restricted one lacking complete virus replication. Moreover, the frequency of infected cells in tumour biopsies was placed at only 7 in 2000 by in vitro co-culture techniques (Calnek and Madin, 1969). More extensive studies showed, nonetheless, that blood and tumour tissue from naturally occurring MD outbreaks could also induce herpesvirus particles and CPE in CKC (Churchill and Biggs, 1968; Witter et al., 1969). Furthermore, whole blood from some clinically normal birds was found to produce these same effects. Again, the presence of herpesvirus particles in the co-cultivated CKC correlated with the ability of these cells induce MD in vivo (Churchill




and Biggs, 1968). The existence of such carrier birds was confirmed subsequently by Kenzy and Cho (1969) who discovered that healthy, two year-old survivors of experimental MD exposure could transmit the disease by contact to younger birds. The host range of MDV did, however, seem to be limited to avian species since a number of mammalian cell types were refractory to in vivo infection (Churchill, 1968; reviewed by Witter, 1972).

The major problem with the argument for an aetiological role of MDV in MD was that, without exception, the agent responsible for both the in vitro CPE and the in vivo reproduction of disease was highly cell-associated (reviewed by Nazerian, 1980). In the absence of cell-free transmission via herpesvirus particles, definitive evidence for a causative role of MDV was lacking. The alternative remained that MDV might be a fastidious contaminant requiring unusual metabolic conditions present in syncytical foci for complete replication. It was a paradox indeed, that MD a very contagious disease transmitted by an airborne mechanism, and yet, the putative aetiological agent was cell-associated. Description of intracellular, cytoplasmic and nuclear MDV antigens in infected CKC using fluorescinated, convalescent sera from exposed birds provided the technical means whereby the riddle was eventually solved (Purchase, 1969; Nazerian and Purchase, 1970; Spencer and Calnek, 1970). Building on this new in vitro discovery, Calnek and Hitchner (1969) examined fixed sections of a large number of tissues from

diseased birds infected with the JM isolate.

Immunofluorescence tests revealed that although they were present in a number of tissues, MDV specific antigens were seen most commonly and with greatest intensity in the layers of epithelial cells lining the feather follicles. Some chickens were, in fact, MDV antigen positive only at this site. Following these indications, electron microscopy was used to demonstrate that whole, enveloped MDV particles were present in the feather follicle epithelium but not in any other tissues examined (Calnek *et al.*, 1970a). These particles were infectious both *in vivo* and *in vitro* and retained their capacity to induce lymphomas after ultrafiltration (albeit with a drop in titre of more than 100-fold). Confirmation of these results was provided later during the same year by Nazerian and Witter (1970).

By 1970 MDV had fulfilled the criteria listed in Koch's postulates (Koch, 1890) for acceptance as the causative agent of acute Marek's disease. First, the virus had been isolated from affected birds. Second, upon reintroduction into susceptible chickens in cell-free form it induced symptoms of MD and could be reisolated from the newly infected hosts. In his review of Koch's postulates and the modifications they have undergone through the years, Evans provides additional criteria which may be used to strengthen the association of a given virus with a particular disease (Evans, 1976). One of these is successful immunization against the disease with the putative aetiological agent or



some derivative of it. A number of such vaccinations have now been effectively employed against MD on a commercial level in the poultry industry.

Discovery of an apathogenic herpesvirus in turkeys led the way to production of the first vaccine against a naturally occurring neoplastic disease (Kawamura et al., 1969; Witter et al., 1970). The herpesvirus of turkeys (HVT) was found to be serologically related but not identical to MDV. Vaccination with live FC 126 strain HVT gave complete protection against subsequent challenge with virulent strains of MDV (Okazaki et al., 1970; Eidson and Anderson, 1971; Eidson et al., 1974). At about the same time laboratory attenuated versions of avirulent MDV (Churchill et al., 1969a-1969b; as well as avirulent field isolates of MDV (Rispiens et al., 1969) were described and used in vaccination trials with good success (Churchill et al., 1969b; Rispiens et al., 1972). Protection against lymphoma development, however, did not preclude superinfection by virulent MDV (Churchill et al., 1969b, Okazaki et al., 1970).

#### The Identification of the Cellular Origin of Marek's Disease Lymphomas

Once the aetiological agent of Marek's disease was discovered and adequate vaccines had been developed, attention turned towards study of more basic aspects of MDV's interaction with the target cell for neoplastic transformation. The first question asked was "what is the

cellular origin of the neoplastic element in MD lymphomas?" In the case of the Epstein-Barr virus (EBV) which has been associated with Burkitt's lymphoma in man, B-lymphocytes are selectively transformed into immortal cell lines by in vitro exposure to cell-free virus (see chapter on other oncogenic herpesviruses). Unfortunately, no such transformation, selective or otherwise, has ever been achieved in vitro with MDV either in cell-free or cell-associated form. As a result, in vitro techniques have not implicated one cell type as the MDV target cell. In vivo studies were required to establish this identity.

As is the case with all solid tumours, the cellular make-up of MD lymphomas was found to be complex. A mixture of small lymphocytes, lymphoblasts, granulocytes, and macrophage-like cells are usually seen (Payne, 1972). The majority of lymphocytes and lymphoblasts in the tumours were found to bear T-cell markers as measured serologically with heterologous antisera (Hudson and Payne, 1973; Rouse et al., 1973; Powell et al., 1974). Normally the T-cell: B-cell ratios in chicken spleen and peripheral blood are about 56:34 and 60:18 respectively (Rouse et al., 1973). By contrast the percentage of T-cells in MD lymphomas ranged from 75-90%. Studies of this nature cannot establish the T-cell identity of the neoplastic element in a tumour, however. The interpretation that T-cells were present in the lymphomas as part of a host immune response against some other infected and/or transformed cell type is equally

valid. In fact, Rouse and his associates explained their results in this manner. B-cells, at least, could be reasonably excluded from the candidates for target cells though. The data of Payne and Rennie (1970), later confirmed by Fernando and Calnek (1971), had shown that while surgical bursectomy at hatching plus sub-lethal x-irradiation (700R) severely depleted the B-lymphocyte pool, these treatments had no effect on MD incidence. The results were in sharp contrast to those obtained in the avian lymphoid leukosis system where bursectomy prevented tumour development (Peterson et al., 1966).

The development of continuous lymphoblastoid cell lines (LCL) from Marek's disease tumour explants (Akiyama et al., 1973; Powell et al., 1974) provided the tools necessary for more conclusive establishment of the T-lymphocyte as the virus-transformed cell in MD. Tests with heterologous anti-thymus and anti-bursa sera showed these new LCL were 100% positive for T-cell surface markers and negative for B-cell determinants (Payne et al., 1974; Powell et al., 1974; Nazerian and Sharma, 1975; Nazerian et al., 1977). Further proof of the T-cell origin of MD tumours came when severe depletion of thymus-derived lymphocytes from susceptible chickens was found to decrease the incidence of gross tumours following MDV exposure (Sharma et al., 1977b). Combination of surgical thymectomy with sublethal irradiation and anti-lymphocyte serum treatments only reduced the T-cell complement to about one-sixth of its

normal level. Total elimination of all lymphomas and microscopic proliferative foci was therefore, not possible.

Other Pathological Syndromes in Which Marek's Disease Virus is Implicated

The lymphomatous manifestation of MDV infection will, in all likelihood, remain the primary focus of research on this virus for some years to come. Other complications of infection do exist, nonetheless, and will be mentioned briefly here.

The first of these syndromes has been termed transient paralysis by Zander (1957) and described by him and other workers (Willemont et al., 1967; Walker and Gruttan, 1968; Wight, 1968; Kenzy et al., 1973; Schierman and Fletcher, 1980; Witter and Fadly, 1980). Transient paralysis is characterized by paralysis of the neck and legs which lasts usually only 24-48 hours and most individuals recover. Affected birds show mild perivascular cuffing of lymphocytes in the central nervous system. Originally, transient paralysis was only seen in eight-twelve week-old birds (Wight, 1968). More recently certain particularly virulent isolates have caused transient paralysis and an acute cytolytic infection in the bursa and thymus in chicks between 5 and 20 days old (Schierman and Fletcher, 1980; Witter and Fadly, 1980). Genetic control of susceptibility to transient paralysis is associated with the chicken major histocompatibility complex (Schierman and Fletcher, 1980).

Another very interesting observation is that MDV

infection in male chickens is linked to an increased incidence of coronary atherosclerosis (Fabricant et al., 1978a, 1980). Actually, the first description of this disease was made by Patterson and Cotral in 1950, but, of course, they had no knowledge of the existence of MDV at the time. Briefly, chickens exposed experimentally to MDV have been shown by the Fabricants and their co-workers to have an increased rate of atherosclerosis in coronary vessels as compared to uninfected controls. In vitro infection of chicken smooth muscle cells also leads to increased deposition of fat in cytoplasmic vacuoles.

The two disorders listed above have not been observed as frequently as classical or acute MD. This may be due more to a lack of awareness on the part of the average veterinary practitioner than to a truly lower frequency of occurrence. In either case, one should not assume that the incidence of these newer forms will remain static. History has already provided one example of a major change in MD's pathology.

#### B. Natural History of Marek's disease Virus Infection

The exact timing of the stages of MDV's in vivo replication in different anatomical sites following exposure varies. One can, nonetheless, piece together a reasonable sequence of events which precede its release in cell-free form from the feather follicle epithelium (FFE). First of all, the most likely portal of entry is the epithelium lining the lungs (Sevoian et al., 1963; Colwell and

Schmittle, 1968; Calnek et al., 1970b; Purchase, 1970). The normal reservoir of infectious MDV in the environment is dry dust and dander in which the cell-free virus is stable for from three to sixteen weeks at room temperature (Colwell and Schmittle, 1968; Witter et al., 1968b; Beasley et al., 1970). Virus specific antigens have been localized in close apposition to the respiratory epithelium following contact exposure (Purchase, 1970). Effective protection against the disease can be achieved by strict isolation of chickens in buildings with filtered air and positive pressure. One report claims vertical transmission of MDV via the egg (Sevoian, 1968) in a fashion similar to that observed for the avian RNA tumour viruses (Vogt, 1965). This report has never been substantiated. Large scale studies of chicks hatched from the eggs of infected dams show conclusively that when reared in isolation these birds do not develop MD (Cole and Hutt, 1951; Solomon et al., 1970; Solomon and Witter, 1973). Vertical transmission of MDV thus seems unlikely. The efficacy of horizontal transmission does vary depending on the strain of virus (Witter et al., 1968b; Phillips and Biggs, 1972).

The progression of infection within the bird generally occurs more rapidly following experimental injection of virus than after contact exposure. The order of replicative events and sites of virus multiplication are the same, however (Phillips and Biggs, 1972). After contact with the lungs, infection spreads rapidly to other organs. From 1-5



days post-exposure, infection of lymphocytes in the spleen, thymus, bursa of Fabricius and liver appears (Purchase, 1970; Aldinger and Calnek, 1970; Calnek et al., 1970b, Frazier and Biggs 1972, Calnek and Hitchner, 1969; Philips and Biggs, 1972; Calnek et al., 1979). There is no consensus as to the exact order of appearance of MDV in these organs, but some indication is given that the spleen may be the first site affected (Calnek et al., 1970b; Frazier and Biggs, 1972). Calnek, in his 1980 review, refers to the unpublished work of Spencer in which splenectomy delayed expression of MDV infection in the other lymphoid organs (Calnek, 1980). Virus replication in the lymphoid organs is incomplete with only intranuclear, naked virus particles being observed and no cell-free infectious virus evident (Purchase, 1970; Calnek et al., 1970b). One exception to this is given in the paper of Frazier and Biggs (1972) whose electron microscopic study gave evidence for enveloped MDV at the nuclear membrane of a few rare lymphocytes in the medulla of the thymus. Expression of intracellular and membrane viral antigens in the lymphoid organs has been detected by immunofluorescence, immunoferritin labelling and agar gel precipitation techniques (Aldinger and Calnek, 1970; Calnek et al., 1970b; Purchase, 1970; Spencer and Calnek, 1970; Nazerian and Chen, 1973). Although the lymphocytes of these organs and in peripheral blood appear to restrict viral replication in vivo, infectious MDV can be recovered from these cells in vitro by cocultivation on CKC.

Recovery of infectious virus does not always correlate with detection of intracellular viral antigens though (Calnek et al., 1979, 1980).

The most prominent feature of early MDV replication in vivo is an acute lymphocytolysis in the bursa of Fabricius and thymus. Extreme degeneration of the follicular structure of the bursa with loss of cellularity takes place during the first two weeks of infection (Jankowski et al., 1969, 1970; Purchase 1970; Philips and Biggs 1972; Fletcher et al., 1972). A similar degeneration was also evident during the same time period in the thymus with involvement of both the cortical and medullary regions. The capacity of virus isolates to induce this early cytolysis in the thymus and bursa correlates directly with their oncogenicity in vivo (Philips and Biggs, 1972; Calnek et al., 1979). Cytolytic infection in these organs peaks between one and two weeks post-infection and declines thereafter with regeneration of the lymphoid elements taking place. A cell-associated viremia begins during the second week of infection as lymphocytes probably spread the virus throughout the body, (King et al., 1972). This second phase of infection affects epithelial cells in the visceral organs. Focal necrosis of the kidney tubules which is occasionally observed (Fletcher et al., 1971) is an example of this. As mentioned previously the only site wherein full replication of MDV is found is in the keratinizing layers of the feather follicle epithelium (FFE) (Calnek et al., 1970a; Nazerian and Witter, 1970).

Production of infectious virus in the FFE with concomitant viral antigen expression starts one to two weeks after exposure (Kenzy and Biggs, 1967; Colwell and Schmittle, 1968). Lesions composed of proliferating lymphocytes with accompanying macrophages are seen at this time in the surrounding areas of the dermis (Lapen et al., 1971). It has been suggested that these lesions represent a host response against virus insult. Clinically normal birds shed virulent virus and can remain carriers of MDV for over two years after initial infection (Kenzy and Cho, 1969). While vaccination with HVT protects against the early lymphocytolysis and subsequent tumour development, such treatment does not alter MDV shedding from the FFE (Purchase and Okazaki, 1971; Fletcher et al., 1972). In their natural environment early exposure of chickens to MDV is still assured today even after almost ten years of commercial vaccination programs.

The type of infection which has yet to be discussed is, of course, that which is present in MD tumour cells. Normally, tumours become grossly evident at 3-4 weeks of age in susceptible birds (Calnek, 1980). By comparison with early infection of lymphocytes and replication in FFE, markers of MDV's presence in lymphoma cells are more cryptic. Viral antigens are only very rarely detected and usually no virus particles are observed (Nazerian and Burmester, 1968; Ahmed and Schidlovsky, 1968; Purchase, 1970; Calnek et al., 1970b). Again, as in the case of

infected lymphocytes, MDV-DNA is present in lymphoma cells and MDV can be recovered upon co-cultivation with permissive CKC (Lee et al., 1972). Additional details on the status of MDV infection in tumour cells will be presented in the following chapter.

### C. Marek's Disease Virus Strains

Soon after the description of the MD herpesvirus and the serologically related but distinct herpesvirus of turkeys, variability in the pathogenicity of virus isolates was noticed. Some virus strains caused primarily acute MD while others induced classical symptoms and still others were found to be apathogenic. Accordingly, Biggs and Milne examined 25 field isolates of MDV and proposed a three group classification scheme based on the clinical manifestations above (Biggs and Milne, 1972). Numerous strains have now been described and it would be difficult to list them all here. Various authors have listed and compared MDV strains in other publications (Purchase and Biggs, 1967; Ishikawa et al., 1972; Philips and Biggs 1972; Calnek et al., 1979; van Zaane and Gilkens, 1980). Certain prototypical strains exist, however, which appear most often in the MD literature. Of these the JM (low passage), GA, HPRS-16 (low passage) and BC- isolates are the most common acute MD strains. HPRS-B14, HPRS-17 and CVI-988 (low passage) are representative of those isolates capable of inducing primarily classical MD. Certain virulent strains lose their

oncogenic potential with continued passage in vitro in permissive cells (Churchill et al., 1969a). JM (high passage) and HPRS-16 (attenuated) belong to this group. Naturally occurring, apathogenic field isolates have also been observed. These strains, typified by HPRS-24 and HPRS-27 (von Bulow et al., 1975) and SB-1 (Schat and Calnek, 1978a), also belong to the nononcogenic group. In addition to the nononcogenic MDV's several HVT strains are known and these are all apathogenic. The FC-126 strain of HVT is used almost exclusively in veterinary practise and in most laboratory research.

All virulent strains of MDV, whether acute or classical, belong to the same serotype; no serological variation has yet been observed between members of these groups (Calnek, 1980). Three serotypes corresponding to virulent MDV, avirulent MDV and HVT can be distinguished by immunofluorescence and immunodiffusion tests with homologous and heterologous antisera (von Bulow and Biggs, 1975). In most cases serological categories coincide well with the classifications based on pathogenicity. Exceptions do exist, however. Attenuated HPRS-16 belongs to the same virulent serotype as virulent HPRS-16 even though the former is nononcogenic (Ross et al., 1975). In regard to this point, no particular antigenic specificity has yet been associated with oncogenic potential. Temperature sensitive mutants of MDV have recently been described (Witter and Offenbecker, 1979). These should provide a valuable tool for correlating

viral antigens with distinct in vivo and in vitro functions.

Besides the major variation related to oncogenicity for chickens in a general sense, a certain amount of difference between isolates exists in their pathogenicity in males vs. females. Most strains do not show a preference for one sex or the other. In those which are selective, a higher incidence of lymphomas is observed in females than in males (Payne et al., 1976; Fabricant et al., 1978). Little work has been directed towards study of this phenomenon, but sex hormones do not seem to be strongly implicated in the mechanism (Biggs and Payne, 1967). As stated by Calnek (1980), three major reasons for the differences in oncogenicity of MDV strains can be suggested. First, there may be inherent genetic variability between MDV strains in their capacity for primary, neoplastic transformation of lymphocytes. For some isolates such as SB-1 and FC-126 this explanation may be valid. These viruses do not initiate lymphomas even in thymectomized, immunosuppressed hosts (Schat and Calnek, 1978). HVT has, nonetheless, been shown to cause mild lymphoproliferative lesions in the nerves (Witter et al., 1976). Moreover, the lack of oncogenicity of other strains cannot be explained on this basis. Some "nononcogenic" isolates do cause tumours upon inoculation in embryonic or immunologically compromised hosts (Calnek et al., 1977). Unfortunately, no in vitro transformation of lymphocytes has yet been reported for MDV. Lack of cell-free MDV has been a great hindrance to this area of research.

Kaaden's report that transfection of CKC is possible with purified MDV-DNA (Kaaden, 1978) may eventually lead to a more direct comparison of the inherent transforming potential of MDV strains.

A second explanation for strain variation in oncogenicity involves a more indirect mechanism. One of the first manifestations of MDV infection is a cytolytic infection of the primary lymphoid organs. Immunosuppression of the host is an early consequence. Numerous laboratories have reported suppression of responses to mitogens following MDV infection (Gunnar et al., 1972; Theis et al., 1975; Lee et al., 1978b). Also, suppressive effects on antibody production (Purchase et al., 1968; Payne, 1970) have been recorded. That virus strains may differ in their capacity to spread from cell to cell during a cytolytic infection was shown by Schat and Calnek (1978a). They found a direct correlation of focus size in CKC in vitro with in vivo oncogenicity. Inoculation of nononcogenic MDV and HVT does not result in a severe cytolytic infection of the thymus and bursa in vivo (Calnek et al., 1979, 1980). These viruses do infect the lymphoid organs preferentially as do the virulent MDV's. Expression of this infection is simply more restricted. It seems reasonable, therefore, to suggest that some MDV strains may fail to induce lymphomas because they leave the host immune system relatively intact and capable of tumour rejection. The reasons behind this failure to cause immunosuppression are at the moment poorly understood.

The third possible explanation for strain variation is that MDV-transformed lymphoblasts may differ in their capacity for in vivo proliferation depending on the virus isolate they encountered. MD tumour cells may differ in their intrinsic proliferative rates. Such variation exists between lymphoblastoid cell lines in vitro (Shearman, personal communication) although it hasn't been directly correlated with in vivo transplantation. Alternatively, highly virulent MDV's may be poorly immunogenic. Comparison of virulent MDV's and their attenuated derivatives by serological methods has only indicated occasional antigen loss in the attenuated strains (see section on virus-induced antigens). The important antigenic differences might be ones detected only by cell-mediated immunity. Finally, effective immunizations have been achieved using fixed preparations of CKC and MD-LCL infected with virulent MDV (Powell, 1975; Powell and Rowell, 1977; Murthy and Calnek, 1979). This point is difficult to reconcile with the hypothesis that certain MDV strains are virulent due to a lack of immunogenicity. One does have to consider, though, that the temporal relationship between presentation of viral antigen and viral replication is somewhat different between vaccination-challenge experiments and natural infection. In the former, immunization occurs first, followed by virus insult. In the later case, on the other hand, the two processes happen almost simultaneously. The development of immunity might thus be expected to be delayed in relation to



viral replication/neoplastic transformation in natural infection. While the idea that virulent MDV's are not immunogenic at all can be reasonably discounted, the possibility of quantitative differences in this property between virus isolates must still be entertained.

#### D. MDV: General Aspects of Productive and Semi-Productive Infection

##### Introduction

MDV infection of fibroblasts and epithelial cells can be either productive or semi-productive. The former case is characterized by full replication and release of enveloped infectious virus particles. Semi-productive replication which is more common, is a state in which only partial synthesis and assembly of virus is observed. Only naked, intranuclear particles are observed and these are noninfectious. A more detailed description of the virus induced proteins will follow in a subsequent chapter of this review. The molecular biology of the replication of MDV fits the general pattern observed for other B-subgroup herpesviruses. Detailed descriptions of the molecular events involved can be obtained from other reviews (Payne et al., 1976; Andrewes et al., 1980). Only general properties of MDV and its replication will be presented here.

##### Virus Structure

MDV has the usual structure of herpesviruses. It consists of a membranous envelope 150-300 nanometers in

diameter which surrounds a central nucleocapsid measuring 90-100 nanometers across (Churchill and Biggs, 1967; Nazerian and Burmester, 1968; Nazerian et al., 1968; Ahmed and Schidlovsky, 1968; Calnek et al., 1970b; Nazerian et al., 1971). The two are separated by an amorphous substance which appears granular under the electron microscope. The capsid is hexagonal and is composed of 162 capsomeres with icosahedral symmetry. Within the capsid lies an electron dense structure composed of toroidal DNA wound around a central core (Nazerian, 1974). Estimates of the size of the core range from 35 nanometers (Nazerian et al., 1971) to 65 nanometers (Nazerian et al., 1968) in diameter. The envelope is acquired at the inner lamellae of the nuclear membrane (Nazerian et al., 1971; Nazerian, 1974). Particles are usually seen as intranuclear inclusion bodies formed by aggregates of pleomorphic virions. Enveloped particles are only rarely observed in the cytoplasm and this is more common for HVT than MDV (Nazerian et al., 1971).

#### MDV DNA

MDV DNA is a double-stranded molecule of approximately  $1.2 \times 10^8$  daltons in molecular weight. The sedimentation coefficient for the double stranded form is 56s while in alkaline gradients the single-stranded molecule runs with a coefficient of 70s (Lee et al., 1971).

Intact double stranded DNA has a density of 1.705 grams/centimeter<sup>3</sup> which is slightly higher than the value for cellular DNA (1,700 grams/centimeter<sup>3</sup>). Calculation of

the G + C content from equilibrium centrifugation gives a mole/percent of 46 (Lee et al., 1971). Continued in vitro passage of MDV results in the appearance of a defective viral DNA which has a 40% shorter piece of DNA and has a lower density, 1.700 g/cm<sup>3</sup> (Lee et al., 1979). Pathogenicity of virus isolates has not yet been related to the presence of such defective DNA. Studies by Witter and Offenbecker argue against interference by defective particles as an explanation for the poor replication of their mutant MDV and HVT strains (Witter and Offenbecker, 1979).

Originally the DNA's of MDV and HVT were thought to have considerable homology (Nazerian et al., 1973). Better techniques for the purification of viral DNA have now led to the conclusion that if homology exists at all, it is on the order of 1-4% of the genome (Kaascka-Dierich et al., 1978; Hirai et al., 1979; Lee et al., 1979; Nonoyama et al., 1980; Kaascka-Dierich, 1980). As pointed out by Nonoyama and his co-workers, who checked for homology by cDNA hybridization using the Southern blotting technique, this amount of DNA could code for 1.5 to 6 polypeptides of  $5 \times 10^4$  daltons molecular weight. One would have to assume, of course, that the detected homology was not due to matching of small scattered sequences. HVT and MDV do share common antigenic specificities. The regions of minor homology are good candidates for the structural genes of the virus polypeptides responsible for these cross reactivities.

### Replication of MDV in Permissive Cells in Vitro

The virus envelope appears to be required for the initial infection of permissive CKC and CEF. The only site where one finds infectious, cell-free virus in vivo (FFE) is also the only site where enveloped particles are regularly described. Similar conclusions have been reached in the herpes simplex system (Smith, K.O., 1964). Naked MDV nucleocapsids have not proved to be infectious. The exact mode by which MDV enters permissive cells is not known and may be accomplished either by fusion of virus and cell membranes or by viropexis (i.e. pinocytosis). Adsorption occurs within one hour in vitro with the first naked, intranuclear particles being observed as early as 10 hours later (Hamdy et al., 1974). New, enveloped virions have been reported as soon as 18 hours post-infection. Penetration of MDV virions after adsorption is facilitated by the chelating agent, EDTA (Aldinger and Calnek, 1972). As much as 40-50 percent of the adsorbed, input virus used by Aldinger and Calnek was incapable of penetration without the addition of EDTA. These authors postulated that aggregated virions in their inocula were responsible for this observation.

Following penetration viral DNA synthesis takes place in the nucleus. Productive infection requires a DNA polymerase specified by the viral genome. This enzyme is more sensitive to inhibition by phosphonoacetic acid (PAA) than the host DNA polymerases and can be distinguished from them by its physical properties (Boezi et al., 1974; Lee et

al., 1976). A similar enzyme has reported for HVT (Leinbach et al., 1976). Phosphonoacetate resistant mutants have been described. The mutation responsible for resistance has been shown to effect the binding constant of the viral polymerase for PAA (Lee et al., 1978a; Lee et al., 1980). A thymidine kinase is also coded for by the MDV genome (Kit et al., 1974). Although enveloped virions are occasionally seen in the cytoplasm of infected monolayer cells, cell-free virus is extremely rare. Secondary foci are not observed even in liquid cultures without agar overlays. Also, addition of virus neutralizing antibodies to cultures after infection does not effect the progressive enlargement of individual foci (Churchill, 1968). These facts would seem to indicate that in culture infection is spread via intracellular bridges rather than by cell-free virus. The net effect of in vitro infection of fibroblasts or epithelial cells is formation of distinct foci of rounded refractile cells. Polykaryocytosis is a common feature (Nazerian et al., 1970). Productive infection in vitro invariably leads to cell death as outlined by Roizman (1972). Necrosis in the primary lymphoid organs following MDV infection may be indicative of a similar process in vivo.

#### E. MDV Infection in Lymphocytes, Tumour Cells and Lymphoblastoid Cell Lines

In contrast to productive and semi-productive infection, a more limited expression of the virus genome is

seen in lymphocytes. Virus particles naked or otherwise, are very rarely found in circulating lymphocytes and tumour cells in vivo (Calnek and Madin, 1969; Calnek et al., 1970a). The frequency of cells containing recoverable virus in a lymphoid tumour is only about 2 in  $10^5$  as measured by co-cultivation on CKC. As will be discussed in the next chapter, the frequency of cells positive for virus membrane antigen is also quite low while cells expressing the so-called Marek's disease tumour associated surface antigen (MATSA) are very often seen (Witter et al., 1975). Latent infection of lymphocytes (infection in which the viral genome is present but no obvious transcription products) is possible. As Calnek points out, however, expression of MATSA in nonproductive infections may mean "a truly latent infection does not occur in MD" (Calnek, 1980).

Marek's disease virus DNA is present in MD tumour cells in vivo (Nazerian et al., 1973). The amount varies from bird to bird, but not very much between tumours in a given individual (Lee et al., 1975). The number of copies reported ranges from 22-80. Most of what is known about the intracellular state of the MDV genome comes from experiments done with lymphoblastoid cell lines in vitro. LCL which produce virus as well as nonproducer lines have been developed (Akiyama et al., 1973, 1974; Powell et al., 1974; Stephens et al., 1976; Nazerian et al., 1977; Hahn et al., 1978; Munch et al., 1978). As is the case in vivo, MD lymphoblastoid cells are rarely positive for virus membrane

antigens or infectious virus. Nonproducer LCL are not positive at all while producer lines show a maximum frequency of virus producing cells of 1-2% at any given time. Regardless of whether cells are replicating virus virtually 100% of the cells in both types of cell lines express MATSA on their plasma membranes. The amount of virus DNA again varies from line to line, but producer LCL, such as MSB-1, have on the order of 60-90 genome equivalents (Nazerian and Lee, 1974) while nonproducers of which MKT-1 is an example, have about 3-15 copies (Tanaka et al., 1978).

The exact state of the MDV genome in LCL is still controversial. Tanaka et al., (1978) reported that 80-90% of MDV-DNA in the MSB-1 line was in a circular episomal state. They claimed that the 10-20% which could not be dissociated from high molecular weight cellular DNA could easily have been an artifact caused by their preparative technique. Kaascka-Dierich and her co-workers examined the DNA extracted from the producer MSB-1 line and the nonproducer HPRS-1 line by isopycnic centrifugation and arrived at a different conclusion. MDV-DNA was present in both integrated and free states in MSB-1 cells while HPRS-1 had only integrated viral DNA (Kaascka-Dierich et al., 1979, 1980). The discrepancy in results from these labs might be explained by differences in the LCL or analytical techniques each employed. Of relevance to this question is the paper by Nazerian and Lee (1976) who reported the effects of phosphonoacetic acid (PAA) on MDV DNA replication in MSB-1

cells. Treatment with PAA inhibited virus production and lowered the number of copies, but did not eliminate the MDV-DNA present. The hypothesis was proposed that maintenance of the integrated viral genome was under the control of a host DNA polymerase. Episomal DNA necessary for de novo synthesis of virus structural proteins was postulated to require a virus-induced enzyme for replication.

To date no characteristic alteration in chromosomal structure has been evident in MD tumour cells. A wide variety of chromosomal abnormalities ranging from aneuploidy to chromosome breakage is apparent in vivo and in vitro (Yoon et al., 1976; Takagi and Sasaki, 1977). In contrast to the Epstein-Barr virus-associated malignancies where an 8 to 14 translocation occurs (Klein, 1979), no single morphological change is consistently found in MD.

#### F. MDV-Induced Antigens

At least six lines of precipitation were observed by Churchill and co-workers in early immunodiffusion studies of MDV antigens in CKC infected in vitro with the HPRS-16 virulent strain (Churchill et al., 1969a). These authors used convalescent sera from chickens having recently survived exposure to MDV. Almost all studies to date have utilized this type of reagent for analysis of MDV-induced antigens. The notable exceptions to this are experiments conducted on the Marek's disease tumour associated surface



antigen in which hyperimmune rabbit sera are most commonly used. As far as this author is aware, no monoclonal antibodies have as yet been reported in the MD literature. Future application of this new technology should aid considerably in purification and characterization of MDV-induced antigens. Lacking such tools, Churchill et al. found that only three precipitin lines were visualized reproducibly with hyperimmune sera. The corresponding antigens were designated A, B, and C. The A antigen is released into culture supernatants while the B and C antigens are cell associated. A and B antigens are shared between MDV and HVT while C antigen is MDV specific (Ross et al., 1975). HVT specific antigens are also indicated by spur lines in precipitin test comparisons of the two viruses with antiserum raised against HVT (Ross et al., 1975). The chronological order of A, B, and C antigen synthesis and expression has not been well studied.

#### The A Antigen of MDV

The A antigen has been analyzed biochemically in detail by at least three groups (Ross et al., 1973; Long et al., 1975a, 1975b; Van Zaane and Gilkens, 1980). Purification was achieved by sequential steps of ion exchange chromatography, isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Owing, perhaps, to a lack of complete purification, there is some disagreement about the exact molecular weight. Long et al gave an estimate of 44,800 daltons while the other groups placed the figure at

60-90,000 daltons. The A antigen is a glycoprotein that is present intracellularly in infected cells but is found in greatest quantity in culture supernatants of semi-productively infected monolayers (Ross et al., 1973; Long et al., 1975a). A antigen is heterogeneous in charge with an average pI of 6.68 (Long et al., 1975b).

Originally the A antigen was thought to be an important factor in determining oncogenicity since this glycoprotein was lost with culture attenuation HPRS-16 to an avirulent form (Churchill et al., 1969a). In fact, HPRS-16 (attenuated) was the first strain used as an MDV vaccine (Churchill et al., 1969b). Linkage of the A antigen with oncogenicity in these experiments was subsequently shown to have been coincidental. Some oncogenic isolates of MDV do not produce A antigen while other nononcogenic viruses such as HVT do (Purchase et al., 1971a). Also, continued in vitro passage of virulent MDV can, in some instances, result in loss of oncogenicity without any detectable serological alteration (von Bulow, 1971).

At about the same time as the A antigen was discovered by immunodiffusion, Chen and Purchase (1970) detected a virus membrane antigen (VMA) on the surface of unfixed CKC infected with the virulent RPL-39 strain of MDV. Analyses with similar immunofluorescence techniques soon confirmed this observation and extended it to other MDV strains and HVT (Ahmed and Schidlovsky, 1972; Ishikawa et al., 1972). The latter authors noted that VMA expression did not require

viral DNA synthesis but was sensitive to puromycin and, therefore dependent on de novo protein synthesis. Mikami et al (1974) claimed to have evidence for another VMA produced later during the course of infection after viral DNA synthesis. Their experiments were done with fixed cells. It is, therefore, unclear whether the antigen they described is actually represented on the cell surface or is located at an intracellular site. MDV VMA is found on productively infected or semi-productively infected non-lymphoid cells in vivo and in vitro. The presence of intranuclear virus particles is strongly but not invariably associated with VMA expression (Ahmed and Schidlovsky, 1972; Nazerian and Chen, 1973). This antigen is seldom observed on infected lymphocytes and lymphoblasts (Calnek, 1980).

Nazerian suggested in his 1980 review that VMA and A antigen may be identical. Unfortunately, there is no direct evidence for this since no truly monospecific antisera against these antigens have been available so far. Using convalescent sera and hyperimmune, rabbit anti-MDV sera. Nazerian has shown that both antigens are lost more or less simultaneously after the 21st passage in vitro in CKC. He also has suggested that VMA is also a component of the virion's envelope (Nazerian and Chen, 1973). Again, conclusive evidence is lacking. Antisera produced in chickens against purified plasma membranes of HVT and MDV infected chick embryo fibroblasts do have neutralizing activity against cell-free HVT (Kaaden and Dietzschold,

1974). Biochemical analysis of these membranes showed the existence of at least two distinct, virus-induced polypeptides. Moreover, 2-D gel separations of whole extracts from such cells reveals at least eight viral proteins (van Zaane and Gilkens, 1980). Similarly, the virus particles themselves are composed of at least eight different polypeptides (Chen et al., 1972). Two of these, VP11 and VP14 are lost after NP-40 treatment and would seem to be part of the virus envelope. No attempt was made in these studies to compare VP11 and VP14 to the classical A antigen or VMA by immunodiffusion.

To summarize it would appear that plasma membranes of MDV-infected CKC and the virus envelope have antigenic determinants in common since antisera against the former will neutralize virus. Both the infected cell membrane and the MDV envelope have at least two distinct viral polypeptides, but whether both or only one of these is shared is not known. Also, whether the A glycoprotein is identical to one of them is unclear but has been inferred indirectly. Monospecific antisera will be needed to resolve this issue. Theoretically, such reagents could be produced by immunization of chickens with infected cell membranes and virions from mutant strains lacking one or more virus polypeptides. This approach assumes, of course, that the required mutations are not lethal and can be selected for - both of which are open questions. Given the available hybridoma technology and the demonstrated capability of

separating virion components on 2D gels, it would seem that production of monoclonal antibodies against wild type MDV would be a more fruitful alternative.

#### The MDV B and C Antigens

As far as is known, the MDV B and C antigens of MDV are always expressed together. This observation is true for both virulent and avirulent MDV strains. The B and C antigens are found both in the nucleus and in the cytoplasm of productively and semi-productively infected cells. The B antigen is found in the cell membrane (Velicer *et al.*, 1978). The C antigen has been studied in only cursory fashion and its biochemical nature is not known. B antigen was reported by Velicer *et al.* (1978) to be a glycoprotein of 58,250 daltons in molecular weight with an isoelectric point of 4.54. As with the A antigen, studies have been hampered by the logistics of producing enough of the material for biochemical characterization. The B and C antigens are analogous to the early antigens (EA) described in Epstein-Barr virus - transformed cells (reviewed by G. Miller, 1980). They do not require de novo synthesis of viral DNA and their expression is enhanced by treatment with iododeoxyuridine (Nazerian, 1975; Dunn and Nazerian, 1977, Silver *et al.*, 1979). MDV EA antigens have been found by the above authors in both producer and nonproducer LCL. In semi-productive infections these antigens can be seen by immunoferritin electron microscopy only in cells with virus particles (Nazerian and Purchase, 1970). In the nonproducer

cell line, MKT-1, EA antigens are induced by iododeoxyuridine. Their synthesis correlates with an increase from 15 to 42 percent in the amount of the MDV genome represented as complementary messenger RNA (Silver et al., 1979). Only 60-70% of this mRNA is associated with the polyribosomal fraction. These data have been interpreted as evidence for both transcriptional and post-transcriptional controls on EA antigen synthesis. Whether the 30-40% of viral mRNA which was not found in polyribosomes is polyA mRNA or actually codes for viral polypeptides is unknown at this time.

The biological functions of the B and C antigens have not been established. MDV does induce at least two enzymes during infection, a DNA polymerase and a thymidine kinase. In view of the probable order of their appearance in relation to viral DNA synthesis (ie. prior to it), they represent possible candidates for MDV early antigens. Technologies already exist for purifying these enzymes which retain their activities in cell-free systems (Boezi et al., 1974; Lee et al., 1976). If monospecific antisera against B and C antigens were readily available, one could attempt to inhibit these enzymatic activities with such antibodies as one means of defining B and C antigen function.

#### MATSA

Powell and colleagues were first to suggest that a serologically detectable tumour associated cell surface marker might exist in the MD system (Powell et al., 1974).

They hyperimmunized rabbits with the HPRS-1 and HPRS-2 MD LCL. Following adsorption on normal cells of various types, antisera were obtained which in immunofluorescence tests stained 96% of the MD LCL cells, 0% of thymus and bursa cells, 1.3% of normal splenic lymphocytes and up to 35% of the cells in fresh biopsies of MD tumours. By contrast, anti-MDV sera raised against infected CKC stained only 1% of the MD LCL cells, 2.8% of cells from MD tumours and 0% of normal lymphocytes from any source. Unfortunately, controls with mitogen stimulated lymphoblasts and lymphocytes from infected birds along with infected CKC were not included. Given this omission some doubt exists about the specificity of their antisera. For example, their results could be explained by reactivities in their antisera against lymphocyte differentiation or histocompatibility markers on lymphoblasts. Such markers would not necessarily be specific to MD tumours.

The following year Witter *et al.* (1975) made similar observations using hyperimmune rabbit sera prepared against JMV (nonproducer) and MSB-1 (producer) cell lines. Again, virtually all the cells of these lines were positive for the antigen they named Marek's disease tumour associated surface antigen (MATSA). Titration of the two antisera against each line showed that highest reactivity was displayed against the homologous LCL. Heterogeneity in MATSA was suggested as a possible explanation. These workers did include the infected, VMA positive chick embryo fibroblasts as a

control. These cells did not react with the putative anti-MATSA sera in immunofluorescence tests. MATSA was, therefore, not the same as the VMA on fibroblasts. According to these authors, up to 27.3% of cells from MD tumour biopsies were MATSA-positive. In addition, only those cells having the morphology of large lymphoblasts were stained. Reservations concerning the role of reactivities to histocompatibility antigens and differentiation markers must also be held about Witter and company's results.

Hyperimmune rabbit sera prepared in a manner similar to that outlined above have now been used to define "MATSA-like" antigens on other producer and nonproducer LCL (Nazerian et al., 1977; Hahn et al., 1978; Munch et al., 1979). Following infection of susceptible line P chickens with the virulent BC-1 strain, MATSA expression can be found on lymphocytes in the spleen as early as 5 days later. Cells in the thymus and bursa become positive about 2 days after the spleen cells (Murthy and Calnek, 1978). Splenic lymphocytes from infected birds adsorb antiMATSA reactivity against MD LCL, but infected CKC ~~will~~ not. No biochemical characterization of MATSA from either MD LCL or fresh tumour biopsies has yet been published.

There are a number of problems with the designation of MATSA as being transformation or tumour specific. The first of these is the possibility that MATSA as it is currently defined may simply be a histocompatibility antigen.

Immunization of mice with chicken red blood cells is known



to produce a strong, preferential response against the chicken major histocompatibility complex (B-complex, Longenecker et al., 1979). In addition, immunization of mice against MD-derived LCL also leads to a preferential response to polymorphic determinants on these cells including MHC antigens (Shearman et al., 1980). Immunization of rabbits with chicken tumour cells might be expected to evoke similar reactivities. Furthermore, sera from chickens which have regressed MD tumours are not good sources of anti-MATSA antibodies (Witter et al., 1975). Also, it is difficult to produce a strong humoral response to MATSA by immunization of chickens with syngeneic lymphoblastoid cells (Nazerian, 1980). The serological identity of MATSA changes from one cell line to another as well. von Bulow and his colleagues could detect no MATSA common to the four MD LCL they tested (von Bulow and Weiland, 1977; von Bulow and Schmid, 1980). They argued that MATSA heterogeneity could be explained entirely on the basis of shared histocompatibility antigens or lymphocyte specific markers on MD cell lines. One group claimed that this was not the case since pooled, normal lymphocytes did not completely adsorb anti-MATSA activity against MD LCL (Sugimoto et al., 1979). Others were unable to completely remove reactivity of hyperimmune sera to a nonproducer MD cell line by adsorption with normal lymphocytes (Sharma et al., 1977a). In the latter case, the cell line and the lymphocytes used for adsorption supposedly shared the same B-complex alleles. In my opinion, neither of

the latter two groups showed conclusively that the normal lymphocytes they used actually did bear all of the same histocompatibility markers as the LCL tested. Hence, additional unadsorbed, anti-histocompatibility antigen reactions could still have been responsible for their results.

Another problem with the usage of MATSA as a marker specific to neoplastic transformation is that nononcogenic MDV and HVT have also been reported to induce the antigen in vivo on lymphocytes (Schat and Calnek, 1978c; Powell and Rennie, 1980b). Witter and his associates have suggested that HVT may have a capacity for "limited" transformation of lymphocytes in vivo. To support this idea evidence has been presented for mild lymphoproliferative lesions in the peripheral nerves and gonads following HVT infection (Witter et al., 1976). However, MATSA was not detected in the lesions reported. As pointed out by Calnek in his 1980 review, another equally valid interpretation of MATSA induction by HVT and nononcogenic MDV is possible; MATSA may not be specific for neoplastic transformation, but rather, may only be an indicator for the infection of lymphocytes by MDV and HVT.

#### G. The Role of MDV-Induced Antigens in Immunity to MD

That the host's immune response is an important factor in the success of vaccination against MD lymphomas is now well accepted. Vaccination protection is not acquired in

chickens immunologically compromised by cyclophosphamide treatment (Purchase and Sharma, 1974; Payne *et al.*, 1978b) or neonatal thymectomy plus low-dose cyclophosphamide injection (Schat and Calnek, 1978b). Also, susceptible birds can be fully protected by inoculation of virus-related immunogens free of infectious virus and viral DNA (Kaaden *et al.*, 1974). This finding argues against a critical role of defective, interfering particles in vaccination. The relative importance of each component of the immune system in protecting against MD will be discussed in more detail in the next section of this review. For the moment, only *in vitro* correlates of the host's immune response will be dealt with in instances where they aid in understanding the role of particular viral antigens in protective immunity.

The vaccination trials which have been conducted to date have employed live virus; fixed, whole cells; plasma membranes; or whole extracts of infected cells as sources of antigen. Attempts to immunize with purified preparations containing only one antigen have not been reported. Direct correlation of the protection observed with the presence or absence of classical A, B and C specificities in the immunizing inocula have not been presented. The one exception to this is that successful vaccination with the A antigen-negative HPRS-16 (attenuated) strain clearly indicates this particular antigen is not necessary for induction of preventive immunity (Churchill, 1969a). Titres of serum neutralizing antibody against MDV have been

directly related to better survival, but again, these antibodies have usually not been examined for reactivity against the A, B and C antigens specifically. Indeed, when titres of precipitin antibodies have been checked, they do not correlate with survival (reviewed by Calnek, 1980). Antibodies to the B and C markers are induced by vaccination and challenge (Churchill *et al.*, 1969a; Velicer *et al.*, 1978), but Kaaden and Dietschold (1975) have shown that immunization with such intracellular MDV antigens is not effective against MD.

The above considerations notwithstanding, one can distinguish between the protection elicited by two general classes of immunogen, viral antigens and tumour or lymphocyte associated antigens. In simplest terms, either type of antigen is protective on its own. The technical basis for all the experiments from which this conclusion is drawn is similar. Preparations of live virus, semi-productively infected monolayer cells, or MD LCL are inoculated into young chicks. These birds are subsequently challenged with either virulent MDV or transplants of nonproducer MD cell lines. On the basis of the antigenic similarities of each immunizing inocula and challenge agent, specific roles in vaccination have been postulated for viral and tumour antigens.

An effective defense against MD can be achieved by prior immunization with glutaraldehyde-fixed HVT-or attenuated MDV-infected fibroblasts (Kaaden and Dietschold,

1974). Similar observations have been recorded with purified plasma membranes and detergent extracts of HVT and attenuated MDV-infected CEF and CKC (Kaaden et al., 1974; Lesnik and Ross, 1975). Since these cells do not exhibit MATSA but do have high levels of VMA, the conclusion has been that immunity to viral antigens alone is advantageous. It could be assumed that live HVT or avirulent MDV protect exclusively by this mechanism, however, this is probably not the case. Live vaccines protect not only against MD but also against the VMA-negative, nonproducer JMV transplant (Mason and Jensen 1971; Sevoian and Weston, 1972; von Bulow, 1977; Schat and Calnek, 1978a; Powell and Rennie, 1980b). In contrast, immunization with VMA-positive, fixed CKC (free of infectious virus) protects against MD, but not against the VMA-negative transplant. One explanation is that live viruses induce immunity in vivo to a tumour antigen on JMV cells. HVT and avirulent MDV's have been reported to cause MATSA expression during infection (Powell and Rennie, 1978, 1980c; Schat and Calnek, 1978b). Immunization with fixed, infected kidney cells would not be expected to elicit an anti-JMV response since such CKC are negative for the putative tumour antigen MATSA. This concept of dual antiviral and antitumour immunity was first proposed by Powell and Rowell (1977). Under their scheme an early antiviral response is thought to limit replication of virulent MDV. The onset of neoplastic transformation is thereby delayed significantly so that the animal has

sufficient time to mount an effective antitumour response. In support of this idea, inoculation of fixed, MDV infected CKC prior to challenge with MDV does lower early cell-associated viremia (Powell and Rowell, 1977; Murthy and Calnek, 1979). The frequency of MATSA-positive lymphocytes in peripheral blood is also decreased. Inoculation with fixed, VMA-negative MD lymphoblastoid cells on the other hand, does not alter early viremia but does diminish the number of MATSA-bearing cells in the blood and does protect from MD (Powell, 1975; Sugimoto *et al.*, 1978; Murthy and Calnek, 1979). A central issue in the interpretation of the facts given above is whether or not infected CKC are truly negative for tumour-associated antigen(s) and MD LCL are entirely lacking in VMA. At least one report indicating shared antigens between infected CKC and a nonproducer cell line has been published (Powell, 1978).

Most of the data in the literature are, nonetheless, compatible with the concept of dual anti tumour and/or antiviral immunity. The exact nature of the antigens involved has not yet been determined though. The tumour antigen involved may not be the serologically defined MATSA. A number of authors have, indeed, questioned the relevance of MATSA and postulated that other, as yet undefined, tumour markers might be more important to development of a prophylactic response (von Bulow, 1977; Schierman and McBride, 1979; Powell and Rennie, 1980b; Schat and Calnek,

1980). The relevant antigens on lymphoma cells may not be serologically detectable. Ross has reported an antigen on infected leukocytes which is serologically silent and only detected by killer T-cells from infected birds. For the most part, in vitro correlates of cell-mediated immunity have provided little in the way of reliable information about such antigens.

It has been shown that HVT and MDV (attenuated) vaccinated chickens develop T-lymphocyte dependent immunity to MD cell lines. A number of experimental systems have been used which employ radiolabelled LCL as targets for in vitro killing (Powell, 1975; Aldinger and Confer, 1977; Sharma et al., 1977, 1978, 1979; Dambrine et al., 1978, 1980; Kato et al., 1980; Schat and Calnek, 1980). Specific killing of MD LCL compared to cell lines transformed by other viruses has been repeatedly observed. Evidence for marginal delayed type hypersensitivity (DTH) reactions (Donahoe, 1980) and antibody-dependent cell-mediated cytotoxicity (ADCC, Schat and Calnek, 1978d) in vaccinated birds has also been published. From these experiments numerous claims have been made that MATSA was the antigen being recognized in in vivo immune surveillance. The data don't allow one to draw this conclusion for a number of reasons. First, Powell (1975) observed no correlation between in vitro CMI and survival. In fact, no such relationship has yet been reported. Second, the maximal percent specific release of radiolabel in all the above listed reports was only on the order of 10%.

Third, cell lines which are syngeneic with the killer cell donor were not used. The responses could, therefore, have been directed in part towards histocompatibility antigens. Fourth, Danbrine et al (1978, 1980) could observe specific in vitro killing only when vaccinated birds were also exposed to virulent MDV. Furthermore, specific lysis was only evident in chickens which eventually succumbed to tumour growth. Fifth and most damaging to the proponents of MATSA as a tumour rejection antigen, Schat and Murthy (1980) observed no decrease in specific in vitro CMI to MSB-1 cells after enzymatic removal of MATSA. Coating of the target cells with anti-MATSA antibodies also had no effect. Thus, if a tumour rejection antigen exists in this system it is more than likely not the one defined serologically as MATSA.

#### H. Major Components of the Immune System and Protection Against MD

##### Introduction

As mentioned previously, the host's immune response plays a major role in vaccinal prophylaxis. In addition, the immune system is an important factor determining survival in unvaccinated birds. Experimentally induced immunosuppression increases the severity of MD in these animals also.

Information about the nature of protective immunity has come primarily from experiments designed to selectively eliminate either humoral or cell-mediated responsiveness. Studies have indicated that T-lymphocyte dependent cell-mediated



responses are ultimately more important in vivo than humoral responses, although maternal antibody does influence the outcome of infection early on. Relatively little is known about the significance of macrophages, ADCC, natural killer cells and interferon in resistance to MD.

### Humoral Immunity

Soon after the discovery of MDV, reports appeared in the literature that progeny of MDV exposed dams were more resistant to MD than hatchlings from unexposed dams. (Chubb and Churchill, 1969; Ball et al., 1970, 1971; Spencer and Robertson, 1972). It was suggested that passively acquired maternal antibody to MDV was responsible for this effect. Mortality in maternal antibody-positive chicks was decreased compared to controls upon exposure to either classical or acute MDV (Chubb and Churchill, 1969). Ball and his co-workers recorded decreases of up to 35% in MD-specific mortality (Ball et al., 1971). Both cumulative mortality and the mean day of death were affected (Spencer and Robertson, 1972). A major criticism of these early experiments was that since the breeder flocks had experienced significant MD mortality themselves, the resistance of the survivor's offspring might have been due to genetic selection. It is now clear that anti-MDV antibody is by itself protective. Increased resistance has been found in progeny of flocks vaccinated with HVT and non-oncogenic MDV (ie. in the absence of any genetic selection through previous MDV exposure, Eidson et al., 1971, 1972). In addition, the same

effect can be achieved by passively administering to antibody-negative chicks the immunoglobulin fraction of convalescent sera (Calnek, 1972a; Burgoyne and Witter, 1973). Both maternal and injected virus neutralizing antibodies decline to undetectable levels by approximately two weeks of age (Burgoyne and Witter, 1973). De novo synthesis of such antibodies at high levels does not occur until about 1 month of age. Accordingly, antibody-positive chicks challenged at day one post hatching are more resistant than those exposed at three weeks of age (Ball et al., 1970, 1971). The probable mode of action of these antibodies is to limit the effective input dose of virus and to slow its replication and spread in lymphoid tissue. By protecting against early destruction in the lymphoid organs the usual concomitant, immunosuppression, is limited (Calnek 1972a, Burgoyne and Witter, 1973; Payne and Rennie, 1973). As one might expect, passive antibody is most protective when chicks are exposed by contact to low doses of MDV; at high doses of virus resistance of this sort is overcome (Ball et al., 1971; van dem Hagen, 1980).

Of potential importance is the effect of maternal antibodies from vaccinated dams on the efficacy of vaccination of their offspring. Although such antibodies are somewhat protective by themselves, they do not lower the incidence of MD to acceptable levels. Each generation must, therefore, be vaccinated. The problem is that maternal antibodies to HVT limit the effective dose of input vaccine

and can prevent a successful vaccination (Calnek and Smith, 1972; Spencer and Robertson, 1972; Fugère et al., 1980). Use of cell-associated vaccines (which are relatively insensitive to humoral antibody) can overcome this difficulty. For practical reasons injection of high doses of cell-free HVT is the method more commonly employed (Huggelen and Zygarach, 1972; Eidson et al., 1972, 1975; Prasad, 1978; van dem Hagen, 1980).

De novo synthesis of antiviral antibodies by young chicks may influence the course of MD. In general, the titre of virus neutralizing (VN) antibodies correlates directly with increased survival rates (Witter et al., 1971; Calnek, 1972b). Precipitin antibody titres vary independently of VN antibodies and do not correlate with survival. One has to be careful as well in assigning a protective function to VN antibodies for several reasons. First, low titres of VN antibodies may be a result of MDV-induced immunosuppression rather than a condition allowing increased virus replication in lymphoid tissue (Higgins and Calnek, 1975). Second, genetically susceptible chickens do make a good early, humoral response to MDV (Shieh and Sevoian, 1974). Chickens differing in genetic resistance to MD do not necessarily differ in their capacity to make VN antibodies (Higgins and Calnek, 1975). Overall serum levels of IgG do not correspond with genetic resistance either except in extremely immunosuppressed, moribund birds (Krieg and Loliger, 1980). Third, surgical bursectomy of 17 day-old embryos, a

treatment which initially eliminates humoral immunity, does not affect age-related resistance to MD (Sharma, 1974). Surgical bursectomy at hatching coupled with sub-lethal X-irradiation also does not decrease inherited resistance (Payne and Rennie, 1970; Fernando and Calnek, 1971; Shieh and Sevoian, 1975b). Resistance to MD develops slowly with increasing age in some genotypes (Sevoian and Chamberlain, 1963; Anderson et al., 1971; Calnek, 1973; Sharma, 1976) and chemical bursectomy does not impede this process (Sharma and Witter 1975). Finally, neonatal, surgical bursectomy coupled with sub-lethal X-irradiation does not prevent successful vaccination with attenuated MDV (Else, 1974), naturally apathogenic MDV (Schat and Calnek, 1978a), or inactivated viral antigens (Payne et al., 1978a). Recent experiments have shown, however, that humoral antibody may enhance vaccinal prophylaxis (Rennie et al., 1980; Powell et al., 1980c).

The bursectomized animals in the above experiments were unable to mount a humoral response to antigens such as sheep red blood cells, salmonella pullorum and bovine serum albumin. No VN antibodies were observed either. Levels of serum IgG and IgM were virtually nil. The data indicate in a definitive fashion that humoral antibody is not necessary for age, genetic or vaccinal resistance to MD. In some cases, in fact, elimination of the bursa actually enhanced survival (Foster and Moll, 1968; Purchase and Sharma, 1974; Cotter et al., 1975). The reason for this is not obvious,

but removal of blocking antibodies and/or suppressor cells have been suggested as possible explanations (Calnek, 1980).

### Cell Mediated Immunity

The available evidence suggests that thymus-derived lymphocytes are very important in MD not only as targets for virus-induced transformation but also as the host's primary mechanism for immune surveillance. In marked contrast to the aforementioned studies of humoral immunity, selective suppression of T-cell-dependent CMI increases the incidence of MD.

Cyclophosphamide was used in early experiments to study the effect of immunosuppression on Marek's disease pathogenesis. In the chicken, shortly after administration of this drug, both cell-mediated and humoral immunity are suppressed. Thymic function is regained relatively quickly following the initial, drug-induced necrosis while humoral immunity is chronically inhibited (Purchase and Sharma, 1974; Calnek et al., 1977). Purchase and Sharma found that cyclophosphamide treatment inhibited HVT vaccination induced resistance. Their interpretation was that an absence of serum antibody was responsible for this result. Actually, CMI was, no doubt, suppressed as well at the time when they challenged. In a more extensive study Payne and his co-workers treated with cyclophosphamide at hatching and followed this with HVT vaccination two days later. Vaccinated birds were challenged with MDV at various times from 14 to 70 days thereafter. While high mortality was seen

in birds given early challenge, birds with delayed exposure at 35 days or more were significantly protected (Payne et al., 1978b). Successful vaccination corresponded with regeneration of thymus-dependent functions. Humoral immunocompetence was not required. In similar studies neonatal thymectomy prevented vaccination with apathogenic MDV (Schat and Calnek, 1978b).

In addition to its effects on vaccination, thymectomy also abrogates age-related and genetic resistance. Lesion regression in older birds has been quoted as evidence that immune mechanisms are responsible for age resistance (Sharma et al., 1973). Thymectomy plus either cyclophosphamide treatment or sub-lethal irradiation (600R) abrogated age resistance (Sharma et al., 1975) Surgical thymectomy at day one either alone or in conjunction with antilymphocyte serum has been used to implicate T-cells in genetic resistance (Shieh and Sevoian, 1975). One early report was published in which thymectomy alone had no effect on MD (Foster and Moll, 1968). This finding is not really at variance with other reports since in the chicken T-lymphocytes cannot be completely eliminated by surgical means alone. Even with additional treatments of X-irradiation and anti-lymphocyte serum about one-sixth of the normal T-cell complement in the spleen remains intact (Sharma et al., 1977b). The existence of residual T-lymphocytes may also explain why MDV can still induce T-cell lymphomas in such thymectomized animals.

Experiments with low-virulence MDV strains further

strengthen the significance of T-cells in MD protection. MDV strains such as CU-1 and CU-2, while avirulent in normal hosts, can cause tumours in thymectomized but not in bursectomized chickens (Calnek et al., 1977).

It is also true that in vitro CMI to MD LCL is T-cell dependent when spleen cells from vaccinated birds are the source of effector cells (Sharma and Coulson, 1977; Sharma et al., 1979; Kato et al., 1980). Ross (1977) found T-cells to be required for in vitro CMI to infected CKC and lymphocytes in his plaque inhibition assay. Unfortunately, for the reasons previously mentioned the relevance of these in vitro CMI assays to the in vivo situation is in some doubt (reviewed by Calnek, 1980). As far as this author is aware, MD resistance has never been transferred from one bird to another with purified populations of immune T-cells. Shieh and Sevoian (1975a) did increase resistance in one strain combination by transplanting intact thymuses from a resistant genotype into genetically susceptible line.

To summarize, direct support is lacking, but a large body of indirect evidence implicates T-cell mediated immune surveillance against either viral or tumour antigens in host prevention of Marek's disease.

#### Other Mechanisms of Immunity

Not much is known about the role of other cell types in immunity to MD. One might reasonably expect macrophages to function by antigen presentation in CMI and humoral immunity. Macrophages from MDV-infected birds have been

reported to suppress the response of normal spleen cells to the mitogen, PHA, in vitro (Lee et al., 1978b). The same macrophages also reduced the proliferative rate of MD LCL in vitro. Whether the net effect of such macrophages in vivo is a plus or a minus for tumour development is undecided. Differential capacities of macrophages from adult and newborn mice to transmit virus to other cell types has been suggested as a mechanism for age-resistance to herpes simplex-induced encephalitis (Johnson, 1964). So far no macrophage function of this type is evident in MD.

Sharma and Coulson (1979, 1980) reported that cells from nonimmunized, older birds could cause low amounts of specific chromium release from labelled MSB-1 lymphoblastoid cells. The cells responsible for this killing were nonspecific, killing a variety of tumour types. In physical properties they resembled the natural killer (NK) cells described in mice (Herberman et al., 1975, Kiessling et al., 1975). Recently Lam and Linna (1979, 1980) transferred resistance to a nonproducer MD transplant, JMV, from 8 week-old chickens to 1 day-old chicks. The physical characteristics of the cells responsible for the transfer suggested that both NK and ADCC activities were involved. Whether or not rejection of the JMV tumour (which was transplanted across MHC barriers in Lam and Linna's work) correlates with resistance to MD following natural exposure, remains to be seen.



## I. Genetic Resistance to MD

### Introduction

There are, at the present time, two types of genetic resistance to MD for which serological markers have been discovered. The first and most commonly studied form is linked to certain alleles coding for the serologically defined antigens of the chicken major histocompatibility complex (MHC or B-complex). The second type of resistance is associated with the antigenic products coded for by the Ly-4 locus. This type of resistance is not linked to the MHC. Ly-4 determines alloantigens on T-lymphocytes. The patterns of inheritance of these two kinds of genetic resistance are different and the mechanisms whereby they confer protection from MD may differ as well. In addition, the existence of other, as yet undefined genes controlling MD susceptibility has been suggested (Stone et al., 1970; Hartman et al., 1980). It is clear from breeding studies and population analyses that single genes can exert very strong influences on survival after MDV exposure (Cole, 1972; Pazderka et al., 1975b). Other genes may, nonetheless, exert more subtle, modifying effects on MD mortality. In any case, both forms of genetic resistance to MD share two common features. (1) Resistance is against lymphoma development and not against infection per se - although levels of vireamia etc. do vary between genotypes. (2) Resistance is never absolute; both MHC-linked and non-MHC-linked protection can be overcome by high levels of neonatal MDV exposure (Sharma, 1976; Bacon

and Witter, 1980; Hartman et al., 1980; Powell et al., 1980a). Maximal protection requires the combination of genetic selection and vaccination (Gavora et al., 1980).

#### MHC-linked Genetic Resistance to MD

The B-complex has now been firmly established as the chicken MHC equivalent to H-2 in the mouse and HL-A in man (Jaffe and McDermid, 1962; Gilmour, 1963; Schierman and Nordskog, 1963; Gleason and Fanguy, 1964; Miggiano et al., 1974). Its nomenclature, structure and function as well as the biochemical properties of its products have been reviewed by Pazderka et al. (1975a) and more recently by Longenecker and Mosmann (1980).

As mentioned earlier, the possibility for control of MD via selective breeding was known from the time of Hull and Cole's paper on the subject in 1947. Discovery of specific genes conferring MD resistance did not come about until MDV itself was classified as an entity distinct from the avian RNA tumour viruses. The rapid divergence in MD susceptibility after only 2-4 generations of selective breeding observed by Cole (1968) in lines N (resistant) and P (susceptible) indicated that a limited number of genes were primarily responsible for MD protection. Hansen (1967) suggested that the B<sup>21</sup> allele was associated with MD resistance in comparisons with the B<sup>19</sup> allele. That certain B alleles might code for susceptibility was suggested by Briles and Oleson (1971) who noted that the B<sup>1</sup> allele was lost selectively from the gene pool following exposure of

flocks to MDV.

Pazderka and her co-workers found additional evidence for an association between B<sup>21</sup> and MD protection. They reported that Coles line N was uniformly homozygous for B<sup>21</sup> while the susceptible line P was segregating for a number of other B alleles (Pazderka et al., 1975b). Formal genetic proof of the importance of B<sup>21</sup> in MD resistance was first given by Longenecker et al. (1976, 1977) who recorded MD mortality in the F2 generation of a cross between line N and a susceptible line. Concordant results were obtained by Briles et al. (1977) in similar backcross experiments. Moreover, B<sup>21</sup> is also present in high frequency in unrelated, outbred populations of chickens which have survived MDV exposure (Pazderka et al., 1975a, 1975b; Longenecker et al., 1976, 1977). The B<sup>21</sup> linkage group may, therefore, have survival value for the species. In addition to B<sup>21</sup>, other B alleles can be ranked according to the degree of MD resistance/susceptibility they provide. B<sup>2</sup>, B<sup>4</sup> and B<sup>6</sup> carriers are moderately resistant (Longenecker et al., 1976; Bacon and Witter, 1980; Briles et al., 1980) while combinations of the B<sup>1</sup>, B<sup>3</sup>, B<sup>5</sup>, B<sup>13</sup>, B<sup>15</sup>, B<sup>19</sup> and B<sup>27</sup> alleles confer increased susceptibility (Pazderka et al., 1975a, Pevzner et al., 1979; Bacon and Witter, 1980; Briles et al., 1980).

Although the exact mechanism of MHC-linked resistance is unknown, immune response genes (Ir genes) for an antitumour response are implicated. At least four pieces of

evidence suggest this. First B<sup>21</sup>/B<sup>21</sup> birds reject MD tumour transplants more readily than do birds carrying other B haplotypes (Longenecker and Gallatin, 1978). Second, rejection of such transplants in a syngeneic system has been shown to require T-lymphocytes (Calnek et al., 1978). Third, MHC-associated resistance is inherited as an autosomal dominant trait which is consistent with the usual pattern for Ir gene transmission in the mouse (McDevitt and Landy, 1974). MHC-linked genes controlling immune responses to such antigens as dinitrophenol and tuberculin, as well as the synthetic copolymers (T,G)-A-L and G-A-T have been reported in chicken systems (Balcarová et al., 1973a, 1973b; Pevzner et al., 1975; Koch and Simonsen, 1977). A fourth bit of evidence comes from experiments with B<sup>1</sup>/B<sup>1</sup> recombinant chickens in which genetic resistance has been tentatively linked with a known B-complex Ir gene (Pevzner et al., 1979). If MHC-associated MD protection is due to Ir gene effects, these are more than likely not directly due to line-specific differences in VN antibody production. Even if one does not question the relevance of VN antibodies in vivo in MD, the fact remains that the prototypical susceptible line P can mount a reasonably strong, humoral response to MDV (Smith and Calnek 1973; Hong and Sevoian, 1974; Higgins and Calnek, 1975). Also, overall levels of serum IgG do not correspond directly with the degree of resistance in inbred lines (Krieg and Loliger, 1980).

B-complex-linked genetic resistance to one clinical

manifestation of MD does not necessarily confer resistance to other MD symptoms. Schierman and Fletcher (1980) have recently shown that protection from early transient paralysis in the BG-1 line is inherited as a dominant trait linked to the B<sup>1</sup> allele. GB-1 birds are, nonetheless, highly susceptible to MDV-induced lymphomas.

#### Non-MHC-linked Genetic Resistance to MD

Resistance to MD can also be conferred by non-MHC-associated genes. The source for this comes from experiments with line 6 (resistant) and line 7 (susceptible), both of which are uniformly homozygous for the same B<sup>2</sup> haplotype (Pazderka et al., 1975a). These two lines were originally derived by selective breeding from a common ancestral stock in 1939 (Waters, 1945). Again, mortality varies in the resistant line with the dose of virus and method of exposure. Contact exposure results in the lowest mortality. MD-specific deaths can range from 0% to 25% in line 6 (Sharma, 1976). The level of exposure has little effect on the MD incidence in line 7 with mortality of the order of 90-100% being reported (Schmittle and Eidson, 1968; Stone, 1969). There is some disagreement as to the phenotype of F1 animals from a line 6 x line 7 cross. Schmittle and Eidson (1968) found that F1's were intermediate with a percent MD incidence of 48% if exposure was by experimental injection and 27% if exposure was by contact. Stone and his colleagues, on the other hand, suggested in a pair of abstracts that resistance was

dominant but influenced by at least three gene loci (Stone, 1969; Stone et al., 1970). Unpublished data of Dr. B.M. Longenecker with reference to      stock are consistent with those of the former      hors. Intermediate susceptibility of the F1 of crosses between other chicken lines has also been reported (Zeitlin et al., 1972; Hartman et al., 1980) with multiple genes being suggested as a possible explanation.

Formal proof that one resistance gene in line 6 and line 7 was not linked to the B-complex was provided by Fredericksen et al., (1977). They repeatedly cross-immunized each line with whole blood from the other genotype. After suitable absorption the resulting antisera were used to define an autosomal locus, Ly-4, which codes for alloantigens on T-lymphocytes (Fredericksen et al., 1977). Serological analysis of a segregating F2 population from a line 6 x B<sup>14</sup>/B<sup>14</sup> cross showed that Ly-4 was not linked to the B-complex.

Ly-4 has two alleles, Ly-4a and Ly-4b, carried by line 6 and line 7 respectively. The Ly-4b allele has been associated with MD susceptibility (Fredericksen et al., 1977; D.G. Gilmour, personal communication). Ly-4a and Ly-4b products are expressed codominantly on F1 cells. In segregating F2 and F3 generations of a line 6 x line 7 cross, Ly-4 heterozygotes are intermediate in MD susceptibility. Parental types (ie. Ly-4 homozygotes) in such segregating populations are again resistant and

susceptible as one would expect on the basis of their Ly-4 phenotypes. The degree of resistance/susceptibility, however, is not as absolute as that found in the original parental lines (Fredericksen et al., 1977; B.M. Longenecker, personal communication). Available information from Ly-4 studies would, therefore, seem to implicate additional genetic loci in the control of MD resistance in line 6 and line 7.

Other information in the literature in regards to actual mechanisms whereby non-MHC-associated genes mediate resistance will be dealt with in the succeeding chapter entitled "Project Rationale".

#### J. Other Herpesviruses Implicated in Neoplastic Disease

##### Introduction

Herpesviruses have been strongly implicated as the causative agents of various cancers of other animals. Epstein-Barr virus (EBV) is thought to be involved in the aetiology of Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). Herpes simplex virus type 2 (HSV-2) may play an important role in the genesis of certain human cervical cancers. Two simian viruses, herpesvirus ateles (HVA) and herpesvirus saimiri (HVS) have demonstrated oncogenic potential in new world monkeys. Other herpesviruses such as the Lucke virus of frogs and the B-lymphotropic herpesviruses of primates also have known or suspected carcinogenic properties. Less is known, however,

in regard to these latter viruses. Hence, the next few sections will deal primarily with EBV, HSV-2, HVA and HVS and the evidence linking them to oncogenesis. More extensive reviews on these viruses have been published recently (Sugden, 1979; Falk, 1980; Miller, 1980; Rapp, 1980; de The', 1980).

### EBV

A possible connection of EBV with the lymphoma described by Burkitt (1958) was evident when herpesvirus particles were discovered in cultured lymphoblasts from biopsies (Epstein et al., 1964; Hinuma et al., 1967). Further support to the theory of an infectious aetiology in BL came when space-time clustering was recorded in "outbreaks" of BL in Uganda (Pike et al., 1967; Williams et al., 1969; Morrow et al., 1971). Subsequently four additional discoveries have been made which make the involvement of EBV in BL as at least a co-carcinogen difficult to question. (1) EBV-DNA is found in multiple copies in Burkitt's lymphoma biopsies (Zur Hausen et al., 1970; Nonoyama et al., 1973) and in cell lines from Burkitt's tumours (Zur Hausen et al., 1972; Adams et al., 1973; Sugden and Mark, 1977). EBV genetic material has also been described by in situ nucleic acid hybridization in NPC biopsies (Nonoyama et al., 1973; Anderson-Anveret et al., 1978). (2) Malignant lymphomas result following experimental EBV infection of new world monkeys (Shope et al., 1973; Werner et al., 1975; Frank et al., 1976). (3) Both fetal and



adult human lymphocytes can be transformed in vitro with EBV to become immortal cell lines (Pope et al., 1968; Sugden and Mark, 1977). (4) A large body of seroepidemiological evidence now exists which shows BL and NPC patients have higher titres of anti-EBV antibodies than do matched controls (Old et al., 1966; Henle et al., 1969, 1970, 1971; Henle and Henle, 1976; de The et al., 1975, 1978). The best evidence of this sort comes from the large scale prognostic study published by de The et al. in 1978. In this project patients' sera were collected both before and after the clinical signs of disease. Those persons with early evidence of significantly elevated antibody titres to the EBV-VCA antigens developed BL much more frequently than low anti-VCA titre controls did. In addition to the putative aetiological function of EBV in BL and NPC, this virus is now firmly established as the causative agent of infectious mononucleosis (IM) a self-limiting lymphoproliferative syndrome of man (Henle and Henle, 1978).

It would, of course, be unethical to formally test the association of EBV with human cancer by all of Koch's original criteria. Perhaps, as Epstein (1976) suggested, the best proof of such linkage would be successful intervention with an EBV-derived vaccine. With the present lack of a suitable means for identifying those individuals at highest risk, such a vaccination program would probably not be feasible for logistic reasons. After all, BL can be controlled reasonably well with chemotherapy (Ziegler et

al., 1970).

Some notable comparisons can be made between EBV and MDV. First, like MDV, EBV is ubiquitous with a high percentage of the human population being infected even in isolated areas (Black et al., 1970; Tischendorf et al., 1970; Also, as with MDV, a large fraction of healthy individuals can be infected. EBV infection per se does not invariably lead to cancer (Chang et al., 1973; Geser et al., 1978). Indeed, other environmental (Burkitt, 1962, 1970; Dalldorf et al., 1964; O'Connor, 1970; Fraumeni et al., 1974; de The, 1977) and genetic (Ho, 1972; Williams and de The, 1974; Purtilo et al., 1977, 1978; Simons, 1978; Kirk et al., 1978) co-factors have been suggested for both BL and NPC. In contrast to the MD model system, the nature of these co-factors is poorly defined. Hopefully, information from MD studies will provide useful parallels which can be developed in EBV research. However, MD lymphomas are of T-cell origin while EBV selectively transforms B-lymphocytes (Jondal and Klein, 1973; Greaves et al., 1975). Direct comparisons between EBV and MDV should, therefore, be made with caution. Of particular interest in this context are the putative receptors by which each virus enters its target cell. Klein's group and others have demonstrated specific binding of EBV to human B-lymphocytes at a site closely associated with the receptor for the third component of complement (Jondal et al., 1976; Yefenof and Klein, 1976; Yefenof et al., 1976, 1977; Robinson et al., 1977). Given the T-cell

character of MD tumours, one might not expect to find such an association in the MDV system. In any case similar efforts to define a receptor in the MD system will be difficult since MDV is more highly cell-associated than EBV.

### HSV-2

Aside from the known capacity of HSV-2 to transform monolayer cells in vitro (reviewed by Nahmias and Norrild, 1980), two lines of evidence implicate this virus in human cervical carcinoma. First, antibodies to HSV-2 have been found in higher titre and greater frequency in women with abnormalities such as squamous carcinoma of the cervix and cervical intraepithelial neoplasia than in matched controls (Rawls et al., 1968, 1969, 1980; Nahmias et al., 1970; Royston and Aurelian, 1970; Adam et al., 1973; Beral, 1974; Skinner et al., 1977; Thomas and Rawls, 1978; Clarke and Anderson, 1979). Gilman et al. (1980) studied the reactivity of these antibodies against specific radiolabelled HSV-1 and HSV-2 polypeptides. Various socioeconomic factors were also taken into consideration in the statistical comparisons of patients and controls. Antibodies to two HSV-2 proteins of 38,000 and 118,000 daltons molecular weight were found more often in patients than in controls.

In addition to such seroepidemiological data, nucleic acid hybridization experiments with HSV-2 specific probes have provided further evidence linking HSV-2 with cervical cancer. In situ hybridization of cloned restriction fragments of HSV-2 DNA to viral mRNA has been demonstrated

in cervical carcinoma cells (Jones et al., 1979; McDougall et al., 1980; Eglin et al., 1980; Wilkie et al., 1980). Hybridization was observed more often in cancer patients than in controls. In a single cancerous cervix cytologically abnormal cells but not normal cells in adjacent areas contain HSV-mRNA (Eglin et al., 1981). Not all fragments of HSV-2-DNA bind equally well to cervical carcinoma cells. According to Eglin and his associates, the location in the HSV-2 genome of those fragments which do bind is coincident with the sites thought to code for the two polypeptides described by Gilman et al. above. Interestingly, a ~~copy~~ of small amounts of HSV-2-DNA in cervical cancer ~~cells~~ (Frenkel et al., 1972) has not been confirmed (Zur Hausen et al., 1974; Pagano, 1975). Hence, the quantity of viral DNA necessary to maintain the transformed state may be minute. In this respect, HSV-2-linked tumours are quite different from EBV and MDV-connected neoplasias. Tumour cells of these other cancers usually contain multiple copies of the entire viral genome.

Again, direct proof of a aetiological role for HSV-2 in human cancer is lacking. Forms of cervical neoplasia may, indeed, exist which have no connection with HSV-2 (Eglin et al., 1981). Given the recent finding of HSV-mRNA in cervical tumours, this virus is, nonetheless, worthy of increased attention in the future.

#### HVA and HVS

HVA and HVS are extremely interesting in that they have

proven lymphomagenic potential in new world primates. HVS was first isolated from a squirrel monkey (Saimiri sciareus; Melendez et al., 1968) while the cross-reacting HVA was isolated from peripheral lymphocytes of spider monkeys (Ateles sp., Melendez et al., 1972). Neither virus causes overt disease in its natural host, but each can induce lymphomas in related new world monkeys (reviewed by Falk, 1980). The tumours induced by HVS are multiclonal (Marcyznska et al., 1973). As with MDV, these viruses appear to be T-lymphotropic (Wright et al., 1976; Falk et al., 1978). Like MDV, HVS is cell associated in vivo (Falk et al., 1972) although relatively high titres of cell-free, HVS and HVA can be produced by in vitro culture techniques. In vivo cell-free virus is seen only in oral secretions of squirrel monkeys (Falk et al., 1973). Horizontal transmission of HVS from squirrel monkeys to owl monkeys with tumours resulting has been reported, however (Barahona et al., 1975). Injection of purified HVS-DNA is also lymphomagenic. Whole HVS virions have been recovered from animals so treated.

Preliminary attempts to vaccinate against the tumourigenic effects of HVS have been undertaken (Laufs, 1974;). In contrast to the routine success of vaccination in the MD system, only moderate protection was achieved in these trials. Also, compared to the chicken system, genetic markers for disease resistance are not well described in simian species. Anyone who has worked with a model system in

which adequate numbers of the subject animals are difficult to obtain can appreciate the obstacles in HVS and HVA research.

## II. PROJECT RATIONALE

At the time the work reported in this thesis began, non-MHC-associated genetic resistance to MD had been studied much less than the MHC-linked type. As described previously, B-complex-connected protection is thought to be a result of Ir gene effects. Hence, there was a tendency among most authors to infer that genetic resistance in line 6 and line 7 was derived from a similar mechanism. Even recent reviewers have claimed that genetic resistance to MD is an immune surveillance phenomenon and is definitely not expressed at the level of a direct MDV-target cell interaction (Calnek, 1980; Nazerian, 1980). The evidence cited most often to support this contention is that chicken CKC and CEF from lines 6 and 7 replicate virulent MDV equally well in vitro (Spencer, 1969; Sharma and Purchase, 1974). Even without taking into account the data which will be presented subsequently in this thesis, I would question the aforementioned interpretation of Spencer, Sharma and Purchase's data for the following reasons. MD is a lymphoma of T-cells, not CEF or CKC. Productive infection of fibroblasts or epithelial cells, a process which may reflect a capacity for infection at the level of the whole animal, is not equivalent to infection or neoplastic transformation of T-lymphocytes and, thus may not be subject to the same genetic restrictions. Beard (1963), who studied genetic resistance to avian RNA tumour viruses, made this point clearly:

"Thus it is evident that the total individual bird is not genetically homogeneous with respect to the sensitivity of different component tissues: myeloid erythroid, lymphoid, renal, and periosteal cells - to respond to a given virus strain. It is clear, then, that the individual represents a population of tissues of different relative susceptibilities varying from one bird to another."

Taking the above arguments into consideration, then, one could postulate in general terms, two mechanisms which might explain the resistance/susceptibility characteristics of line 6 and line 7. (1) Line 6 might differ from line 7 by being more capable of immune surveillance against MDV or MDV-induced tumours. (2) Line 7 might differ from line 6 by some property allowing for increased infectivity, transformability or intrinsic proliferative capacity of the target cell for MDV. These two explanations are not mutually exclusive. The diversity in clinical expressions of MDV infection outlined previously might well imply that the mechanisms of genetic resistance may be complex having several modes of expression even in one animal.

With respect to the above hypotheses several properties of line 6 and line 7 were known which aided in the selection of a working hypothesis. First, susceptible line 7 birds developed a much higher titre of lymphocyte-associated vireamia than did line 6 following neonatal exposure to MDV (Sharma and Stone, 1972; Sharma, 1976; Fredericksen et al.,



1977). This observation was explained equally well by both immune surveillance and target cell hypotheses. Differential infectivity, however, was also demonstrably higher in line 7 embryos at stages of development during which classical immune mechanisms were unlikely to be involved (Longenecker et al., 1975). Second, F<sub>1</sub>'s of a line 6 x line 7 cross were intermediate in MD resistance (B.M. Longenecker, personal communication) rather than resistant as one might expect if dominant Ir genes coded for tumour rejection. Third, in line 7 susceptible birds the frequency of cells capable of mounting a graft versus host reaction (GVHR) was 2.8 times higher than in the resistant line 6. The GVHR is dependent on alloreactive T-cells and susceptibility in other lines had been linked to high GVHR competence (Longenecker et al., 1973). Also, the response of line 7 to various T-cell mitogens was greater than that seen in line 6 (B.M. Longenecker, unpublished data). Both of these facts were suggestive of a larger or more reactive T-lymphocyte pool in line 7 than in line 6. Fourth, serological markers on T-lymphocytes in these lines, namely the Ly<sup>b</sup>4 antigens, had been associated with MD susceptibility (Fredericksen et al., 1977).

The fifth observation about line 6 and line 7 requires a bit more explanation. The basic fact is that no report of a line-specific difference in immune responses against MDV-related antigens in the absence of live virus infection has ever been published. It is true, lower titres of VN

antibodies have been observed occasionally in line 7 than in line 6 (Sharma and Stone, 1972). Unfortunately, it is not clear whether this is due to an Ir gene difference or to immunosuppression from a more extensive early cytolytic infection in the susceptible line. Such data can, thus, be explained by either of the two, aforementioned hypotheses.

In other avian RNA tumour virus systems genetic resistance is known to be expressed at the target cell level (Vogt and Ishizaki, 1965; Crittendon, 1968; Payne et al., 1971; Purchase et al., 1977). In some cases the cellular resistance is almost absolute. In other instances as little as a 2-fold difference exists between resistant and susceptible cells in number of virus "hits" required for transformation (reviewed by, Weiss, 1975).

Overall, in contrast to current dogma, it seemed to us that the available facts about lines 6 and 7 supported the target cell hypothesis, although immune surveillance differences were certainly not ruled out. Adopting as a working hypothesis the target cell explanation, two predictions were made. The first was that line-specific differences in some parameter(s) of MDV infection of lymphocytes in vitro could be found. The second was that line 6 birds could be made more susceptible to MD by adoptive transfer of line 7 target cells. The first experiments reported in this thesis were designed to test these predictions.

### III. MATERIALS AND METHODS

#### Eggs

Hy-Line SC, White Leghorn, fertile hens' eggs homozygous for the B<sup>2</sup> allele at the chicken MHC were obtained from Hy-Line International, Dallas Center, Iowa. Lines 6 and 7 fertile hens' eggs were supplied by the Biosciences Animal Center, University of Alberta. Line 6 and 7 are both uniformly homozygous for the B<sup>2</sup> allele at the chicken MHC. Hy-Line SC eggs were incubated in a Robbins Hatchomatic Incubator, (constant temperature, humidity, periodic rotation, Robbins Incubator Co., Denver Colorado). Line 6 eggs used for thymus grafting were incubated similarly with periodic rotation until day 11. Following grafting, the line 6 eggs were incubated as before except that they were maintained in a horizontal position without rotation.

#### Chickens

Specific pathogen free (SPF) line 6 and line 7 chickens were supplied by the Biosciences Animal Center, University of Alberta. Birds of this flock were determined to be free of MDV neutralizing antibodies at the time these experiments were performed.

#### Viruses

The BC-1 isolate of MDV was maintained in cell-associated form in vitro by serial passage of the MD lymphoblastoid cell line MSB-1 of Akiyama et al. (1973). The FC-126 isolate of HVT was obtained in lyophilized form from

Salisbury Laboratories, Ltd., Scarborough, Ontario. Prior to its use in the FIA and ADRA assays HVT was reconstituted in RPMI 1640 with 5% FBS (Gibco, Canada, Calgary) and centrifuged at 500 g for 15 minutes to remove large cell debris.

#### Immunization of mice

Five CBA/J mice were injected in both hind footpads with 10,000 RFU of HVT infected Hy-Line SC chick embryo fibroblasts (CEF) in Complete Freund's Adjuvant. Fourteen days later an identical injection of HVT was administered in Incomplete Freund's Adjuvant.

#### Fusion of mouse spleen cells with mouse myeloma cells

Three days after the second immunization with HVT-infected CEF, spleen cells from the mice were pooled and fused with MOPC 315.43 cells using polyethylene glycol as described previously by Longenecker et al (1979) who used a modification of the technique published by Galfré and co-workers (1977). MOPC-315.43 was used as a myeloma parent since this clone does not synthesize immunoglobulin heavy chain. An altered lambda light chain is synthesized but is not secreted even in hybrids made with normal spleen cells (Mosmann et al., 1979). The ratio of spleen cells to myeloma cells in the fusions was 10 to 1. After dilution in selective medium cells were seeded into Linbro 24-well, flat-bottomed culture trays (Flow Laboratories Inc., Missisauga, Ontario) at a density of  $10^5$  myeloma cells per ml. Hybrid clones were selected after 2-3 weeks of growth in

RPMI 1640 containing 20% fetal bovine serum, 100 micromolar hypoxanthine, 30 micromolar thymidine, 0.5 micromolar aminopterin, 1 millimolar ouabain. Fresh mouse blood was also added at a concentration adjusted to give  $2 \times 10^7$  mouse red cells/ml. The feeder effect of mouse blood is quite significant (Metcalf, 1973; Mosmann *et al.*, 1979) and eliminates the need for a medium change during clone selection.

#### Screening of hybridoma clones

As a common first step in the two methods used to screen for production of antibodies to HVT, plasma membrane fractions of HVT-infected and uninfected Hy-line SC CEF were prepared. Briefly,  $2 \times 10^8$  CEF were lysed in 2ml. of distilled water by a  $4 \times 10$  second sonication at the maximum setting with a Biosonik IV sonicator (VWR Scientific, San Francisco California). The lysates were partially clarified by centrifugation at 400g for 30 minutes. Supernatants were then centrifuged 90 minutes at 56,000g. The resulting supernatants were discarded. Pellets containing cell membranes were resuspended by sonication as before in phosphate buffered saline (PBS).

Cell membrane preparations were then adjusted to a protein concentration of 100 micrograms/ml. in PBS and coupled to ELISA microtiter plates (Cooke Laboratory Products, Alexandria, Virginia) by overnight incubation at 4°C. Unadsorbed material was removed from the plates by repeated washing with PBS, 100 microlitres of each hybridoma

supernatant was then added to each coated microwell. ELISA assays were performed as previously described by Voller et al. (1976).

For an additional method of screening cell membranes were first adjusted to 1 mg./ml. and covalently coupled to B<sup>2</sup>/B<sup>2</sup> chicken red blood cells (CRBC) with 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide (Mosmann et al., 1980). Coupled CRBC were then used as indicators in hemagglutination assays with hybridoma supernatants as outlined by Longenecker et al. (1979). Briefly, 100 microlitres of hybridoma supernatant was added to each well of a 96 well, V-bottomed microtitre plate (Flow Laboratories). 100 microlitres of a 0.25% w/v suspension of coupled CRBC in RPMI 1640 with 0.5% FBS was then added to each well. After 1 hour at room temperature, wells displaying direct agglutination were recorded. After washing each well 3x with PBS, 100 microlitres of a rat-anti-mouse immunoglobulin reagent (Cappel Laboratories, Cochranville, Pennsylvania) was added to each well to develop indirect agglutination.

Hybridoma clones were considered positive for antibodies to HVT if they met one of the following criteria: 1) supernatant gave a positive color reaction on ELISA plates coated with infected CEF membranes but not those coated with membranes from normal CEF or 2) supernatants directly or indirectly agglutinated CRBC coupled to infected cell membranes but not those coupled to uninfected cell

membranes.

#### Propagation of hybridoma clones from ascites form

$2 \times 10^6$  cells were injected intraperitoneally into CBA/J mice that had been irradiated 900 rads previously, and injected with pristane (2-6-10-14 tetraamethylpentadecane, 0.5ml) at least 2 weeks previously. When the mice were visibly swollen with ascites (1-3 weeks), the fluid was tapped with an 18-gauge needle and the cells were removed by centrifugation.

The ascites fluids were fractionated and partially purified by gel filtration on a Sephadryl-300 column equilibrated with 0.5M NaCl, 0.1M Tris HCl, pH 8.0. Fractions were pooled as indicated in figures 6a and 6b, vacuum-dialyzed to a 2ml. volume and then adjusted with medium to a 2.5mg./ml. protein concentration before use.

#### Virus neutralization tests

Ascites fluids and purified antibodies were diluted as indicated in figures 7 and 8. Indicator cells were secondary CEF prepared from 10 day-old Hy-line SC embryos and propagated in Linbro multi-well trays (8 well). 0.6ml. of antibody diluted with RPMI 1640 with 0.5% FBS was mixed with 0.6ml. of medium containing approximately 300 focus forming units (FFU) of HVT. After 45 minutes of incubation at room temperature, 0.25ml. aliquots were added to each of 4 CEF culture wells, from which the culture medium had been removed. Virus was allowed to adsorb for 45 minutes at 37°C. After adsorption the cultures were washed with PBS

(2x2ml./well) and fresh medium was added. At 120 hours post-infection cultures were fixed by treatment with 0.1% glutaraldehyde for 2 minutes. Foci were counted by light microscopy without staining.

#### Coupling of monoclonal antibodies to fluorescinated latex beads

Carboxylated, fluorescinated latex beads were obtained from Covalent Technology Corporation, San Jose, California. The beads were 1 micron in diameter. Column purified, monoclonal antibodies were covalently coupled to the beads by the carbodiimide reaction (Hoare and Koshland, 1967) as described by Park et al. (1979). The reaction was carried out with antibody at a protein concentration of 2.5mg./ml. which resulted in saturation of available sites on the beads. Coupled beads were stored in RPMI 1640 at 4°C. until use.

#### Immunofluorescence tests

Hy-Line SC secondary CEF cultures were propagated on coverslips in Linbro multi-well trays. Cells were infected with HVT as described for virus neutralization tests. 120 hours post-infection the coverslips were fixed for 30 seconds with 0.1% glutaraldehyde in PBS. Controls included mock-infected cultures. Coverslips were incubated with antibody coupled beads overnight at 4°C. in a horizontal position. Each coverslip was then washed extensively by flooding it in PBS with a Pasteur pipette. A 1 to 1 mixture of glycerol and PBS was used for mounting coverslips on



slides. Fluorescence photomicrography was done with a Zeiss fluorescence microscope (Zeiss Canada, Toronto). A magnification of 200x and an exposure time of 2 minutes were used.

#### In Ovo Assay for Infectious Centres

Infectious centres in spleen and peripheral blood were assayed as described by Longenecker et al. (1975) in Hy-Line SC B<sup>2</sup>/B<sup>2</sup> embryos histocompatible with the MHC of the donor spleen and peripheral blood lymphocytes.

#### Focus inhibition assay (FIA)

This assay for virus adsorption was performed according to the following protocol. Cell free, lyophilized FC 126 HVT was reconstituted in minimum essential medium (MEM) with 0.5% FBS and centrifuged at 500g for 15 minutes to remove large cell debris. Cell suspensions from spleens and thymuses of 3-4 week-old donors or from thymus grafts were prepared by digestion for 45 minutes at 37°C. with a solution of 0.25% w/v collagenase, 100 microgram/ml. dnase (Sigma, St. Louis) in MEM with 0.5% FBS. Cells were then washed 3 times in RPMI 1640 with 0.5% FBS and mixed with HVT. Briefly, 0.5ml. samples containing various numbers of cells were mixed with 0.5ml. aliquots of HVT containing  $5.0 \times 10^2$  FFU of virus (as determined previously on CEF monolayers of Hy-Line SC origin). After incubation for one hour at room temperature the cells were pelleted by centrifugation at 500g for 7 minutes. The virus remaining in the supernatant was then assayed on CEF monolayers of

Hy-Line SC origin. HVT foci were stained with Giemsa stain 72 hours post infection. Each test was done in quadruplicate.

#### Antibody-directed Rosette Assay (ADRA)

The ADRA tests were carried out according to the protocol of Mosmann et al. (1980) with the following modifications:  $10^6$  line 6 and line 7 cells being tested for virus adsorption were first incubated in v-bottomed microwells with HVT for 1 hour at room temperature in 200 microlitres of RPMI 1640 with 5% FBS containing 1000 FFU of HVT. Cells were then washed 3x in medium and mixed at a 20:1 ratio with CRBC coated with an anti-HVT monoclonal antibody (HVT.2). Rosettes were counted after an incubation period of 2 hours at room temperature.

#### Induction of Haemopoietic Chimaerism

$1.5 \times 10^7$  spleen cells from day 15 line-7 or line-6 embryos were injected intravenously into day 15 line-6 embryos which were allowed to hatch. As a test for cellular chimaerism, other  $B^2/B^2$  embryos were injected with embryonic spleen cells from  $B^{15}/B^{21}$  embryos and allowed to hatch. Erythrocytic chimaerism was detected at 4 weeks of age by cellular radioimmunoassay using specific alloantisera (Longenecker et al., 1978). All  $B^2/B^2$  birds which were injected with  $B^{15}/B^{21}$  embryonic spleen cells in this and in several experiments unrelated to this study (Havele, unpublished; Longenecker et al., 1978) were found to be highly chimaeric when tested several times after hatching.

The assumption is made line-6 embryos that have been injected with line-7 embryonic spleen cells are likewise chimaeric, especially since they are histocompatible with respect to the MHC (both lines are B<sup>2</sup>/B<sup>2</sup>) and it is well known that it is much easier to induce chimaerism when an MHC difference is not involved (Takada and Takada, 1971).

#### Thymectomy and thymus transplants

Newly hatched chicks were thymectomised or sham operated as described previously (Longenecker *et al.*, 1966). For the thymus transplants, thymus lobes were cut into 2 or 3 pieces and immediately transplanted subcutaneously lateral to the oesophagus in the areas which were vacated by the removal of the host's thymus lobes. At least one entire thymus equivalent (14 lobes) was transplanted to each recipient. For transplantation of irradiated thymuses, donor chicks recieved 1000r of gamma irradiation 4 hours prior to removal of thymic lobes for grafting. Microscopic examination of cell suspensions from such irradiated thymuses revealed that, at the time of transplantation, few if any cells having the morphology of lymphocytes remained in the grafts.

#### Exposure to MDV in mortality experiments

All chicks were exposed to MDV by housing them next to 4-6 week old chickens that had been inoculated at hatching with 10,000 in ovo lesion-forming units (Longenecker *et al.*, 1975) of the BC-1 isolate of MDV (Spencer *et al.*, 1974) and the day of death from MD was recorded.

### Irradiation of Recipient Chicks in Adoptive Transfer Experiments

Line 6 and line 7 chicks used as recipients in mortality experiments (TABLE IV) and Hy-Line 50 chicks used as recipients in in vivo infectious centre studies (Figures 17 and 19) received 500r of gamma irradiation from a <sup>137</sup>cesium source at a dose rate of 110r/minute on day 3 post-hatching. A second 500r dose was administered on day 8 post-hatching. This irradiation protocol is sublethal but was found to markedly diminish the number of host-specific, MDV infected lymphocytes compared to unirradiated controls upon subsequent natural exposure of unreconstituted chicks to MDV.

### Adoptive Transfer of SPF Spleen Cells into Irradiated Recipients and Conditions of MDV Exposure

Spleen cells used for adoptive transfer in mortality experiments (TABLE IV) and MDV infectious centre tests (Figures 17 and 19) were obtained from 8 week-old line 6 and line 7 donors which had been maintained under SPF conditions. Single cell suspensions of spleen cells were prepared via enzymatic digestion for 45 minutes at 37°C in RPMI 1640 with 0.5% FBS, 0.25% collagenase and 100 microgram/ml dnase (Sigma, St. Louis). Red blood cells and dead cell debris were removed by centrifugation over an Isolymp solution (Gallard Schlesinger Chemical MFG. Corp., New York) for 15 minutes at 400g in a Sorvall GLC-1 centrifuge. Donor cells were then washed twice in RPMI 1640

prior to use. In the experiments presented in Table I, irradiated line 6 and line 7 hosts were injected intravenously 2 hours after the second dose of irradiation with  $2 \times 10^8$  donor spleen cells. These birds were immediately placed under conditions of contact exposure to 10 line 7 age-matched, seeder chicks which had received  $1 \times 10^7$  MSB-1 cells via intraperitoneal injection. The incidence of MDV-specific deaths was recorded for a period of 14 weeks at the end of which significant losses due to MDV had ceased. The irradiated Hy-Line SC chicks used in the experiments of Figure 17 were reconstituted and exposed in a manner identical to that of the mortality experiments. Irradiated Hy-Line SC recipients used in the infectious centres tests in Figure 19 were exposed to MDV immediately following the second dose of irradiation and received  $10^8$  SPF line 6 or line 7 spleen cells 12 days later.

#### Grafting of Thymus Fragments to the CAM

SPF, 4 week-old line 6 and line 7 chicks were exposed to 1000r of gamma-irradiation from a  $^{137}$ cesium source. Four hours later the birds were sacrificed, the thymus glands were removed and minced into 1 mm. fragments. At this time the fragments were found to be depleted of morphologically distinct thymic lymphocytes. Eight to ten individual fragments of thymic stroma were then grafted onto the CAMs of 11 day-old line 6 embryos which had been prepared according to the method employed by Keller et al. (1980) in their studies of haemopoietic colonies on the CAM. Seven

days post-grafting the grafts were removed and treated with collagenase-dnase to yield a single cell suspension. Larger stromal cells were then separated from the lymphocyte fraction by velocity sedimentation at unit gravity for 10 minutes in a 10ml. volume of RPMI 1640 with 5% FBS in 17 x 100mm. Falcon culture tubes (Beckton, Dickinson and Co., Canada, Calgary). Cells were then washed twice in RPMI 1640 with 5% FBS.

#### IV. RESULTS

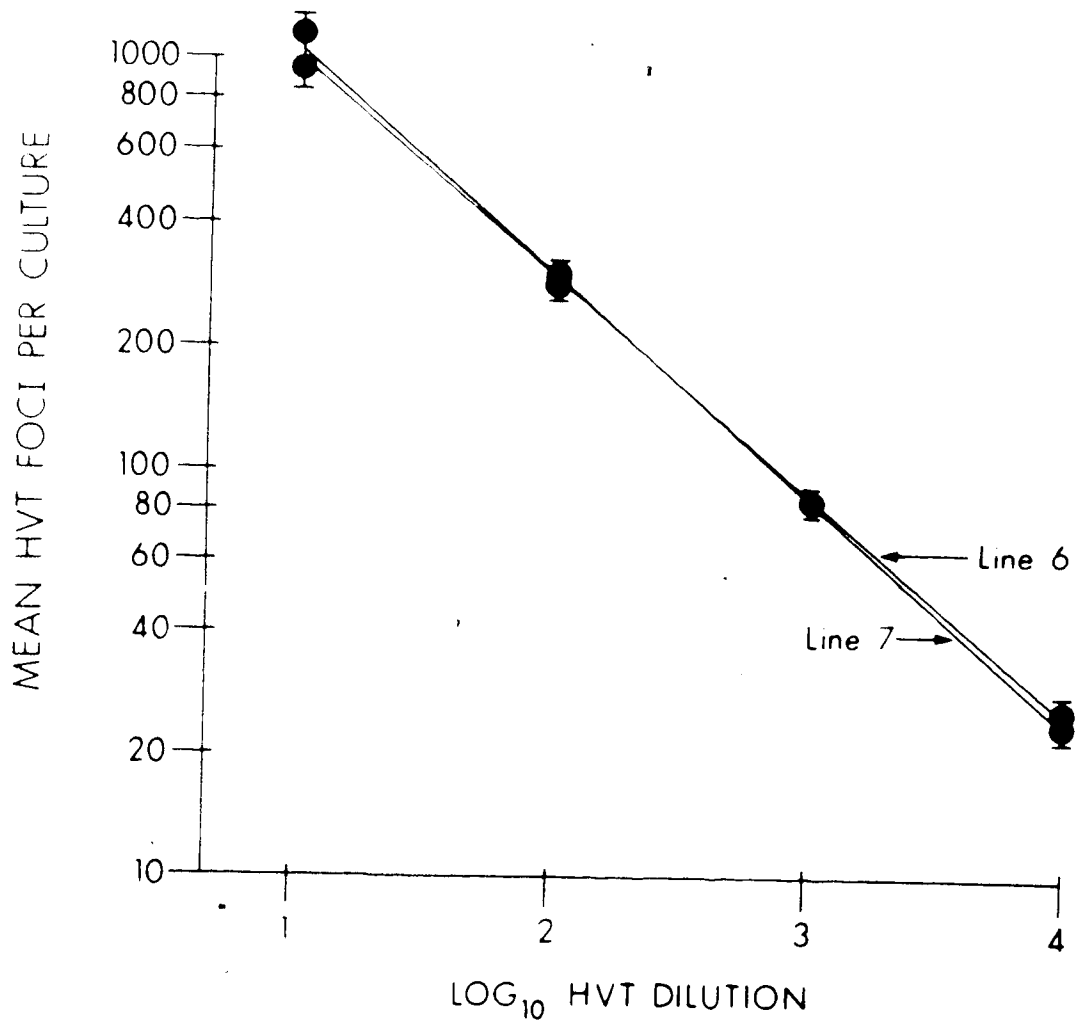
##### A. Infection of Line 6 and Line 7 Cells With HVT In Vitro

One expects, on the basis of the target cell hypothesis, that line 6 and line 7 lymphocytes might differ significantly in their abilities to adsorb and replicate MDV in vitro. As pointed out in the literature review, experiments designed specifically to study this possibility are impractical due to a lack of cell-free MDV. Cell-free HVT, on the other hand, can be produced in sufficient quantity for such experiments. As mentioned previously, HVT is antigenically (and presumably structurally) similar to MDV. The natural history of in vitro infection with HVT strongly suggests that this virus has a cell-tropism similar if not identical to that of MDV (see section of literature review on virus strains). Also, as with MDV, fibroblasts from these two lines do not differ in their capacity to replicate HVT (Figure 1). Therefore, the first experiments which are reported in this thesis utilized HVT as a probe to compare in vitro infection of line 6 and line 7 cells. The use of HVT is, admittedly, a concession to technical practicality; one does have to bear in mind the dissimilarity of MDV and HVT at the genomic level. This reservation notwithstanding, permissivity to HVT infection did prove to be a useful marker; it indicated that line 6 and 7 cells do not respond identically to herpesvirus infection.

Fig. 1 HVT-infection of line 6 and line 7 CEF in vitro. Secondary chick embryo fibroblasts (CEF) were prepared from 10-day line-6 and line-7 embryos and propagated in Linbro multi-well trays. Lyophilised FC126 strain HVT vaccine was reconstituted with medium and centrifuged at 500g for 15 min to remove large cell debris. Four replicate wells were infected with each serial 10-fold dilution of this preparation, the highest concentration of which had yielded  $1.25 \times 10^4$  focus-forming units per well when assayed previously on Hy-Line SC CEF. HVT foci were stained with Giemsa stain and counted at 64 h post-infection. Error bars indicate 1 standard deviation from the mean.



FIG 1 INFECTION OF CHICK EMBRYO FIBROBLASTS WITH HVT



Since adsorption of virus is the first step in infection, the first experiments used a focus inhibition assay (FIA) designed to examine this process. Thymus cells from 4-week-old line-7 and line-6 birds were mixed with HVT, incubated at room temperature for 1 hour, and the cells were pelleted by centrifugation. The amount of infectious HVT remaining in the supernatant was determined by plaque formation on CEF monolayers (Figure 2). It was found that populations of line-7 thymocytes adsorbed a maximum of 20 - 30% more HVT than line-6 thymocytes (Figure 2,  $p < 0.001$ ). While the magnitude of the difference in adsorptive capacity is not impressive, it is nonetheless highly consistent as this experiment has been repeated six times with identical results.

Spleen cells from these two lines were then tested for adsorptive capacity since this organ is known to contain a higher proportion of mature T-lymphocytes, a possible target cell for MDV transformation. At the cell concentrations tested, line-6 spleen cells did not adsorb a detectable quantity of HVT while line-7 spleen cells adsorbed HVT in a dose-dependent fashion (Figure 3). At the highest cell concentration tested ( $240 \times 10^6$ ) line-7 spleen cells adsorbed over 50% of infectious HVT. This experiment has also been repeated six times with consistent results. Following virus adsorption, the number of infected cells of each type was estimated after the cells had been washed and incubated for 24h. Line-7 spleen cells induced 6.7 times

Fig. 2 Adsorption of HVT by line 6 and line 7 thymus cells. HVT was prepared as described in Fig. 1. Cell suspensions from thymuses of 3-4-week-old donors were prepared by collagenase digestion, washed three times in medium and mixed with aliquots of HVT. Only those cells having morphologies characteristic of lymphocytes were counted and included in the calculation of thymus cell number for data shown in Fig. 2. Virus adsorption was carried out at room temperature for 1 h and the cells were then pelleted by centrifugation. The amount of infectious HVT remaining in the supernatant was estimated by focus formation on replicate cultures of CEF of Hy-Line SC origin. Error bars indicate 1 standard deviation from the mean.

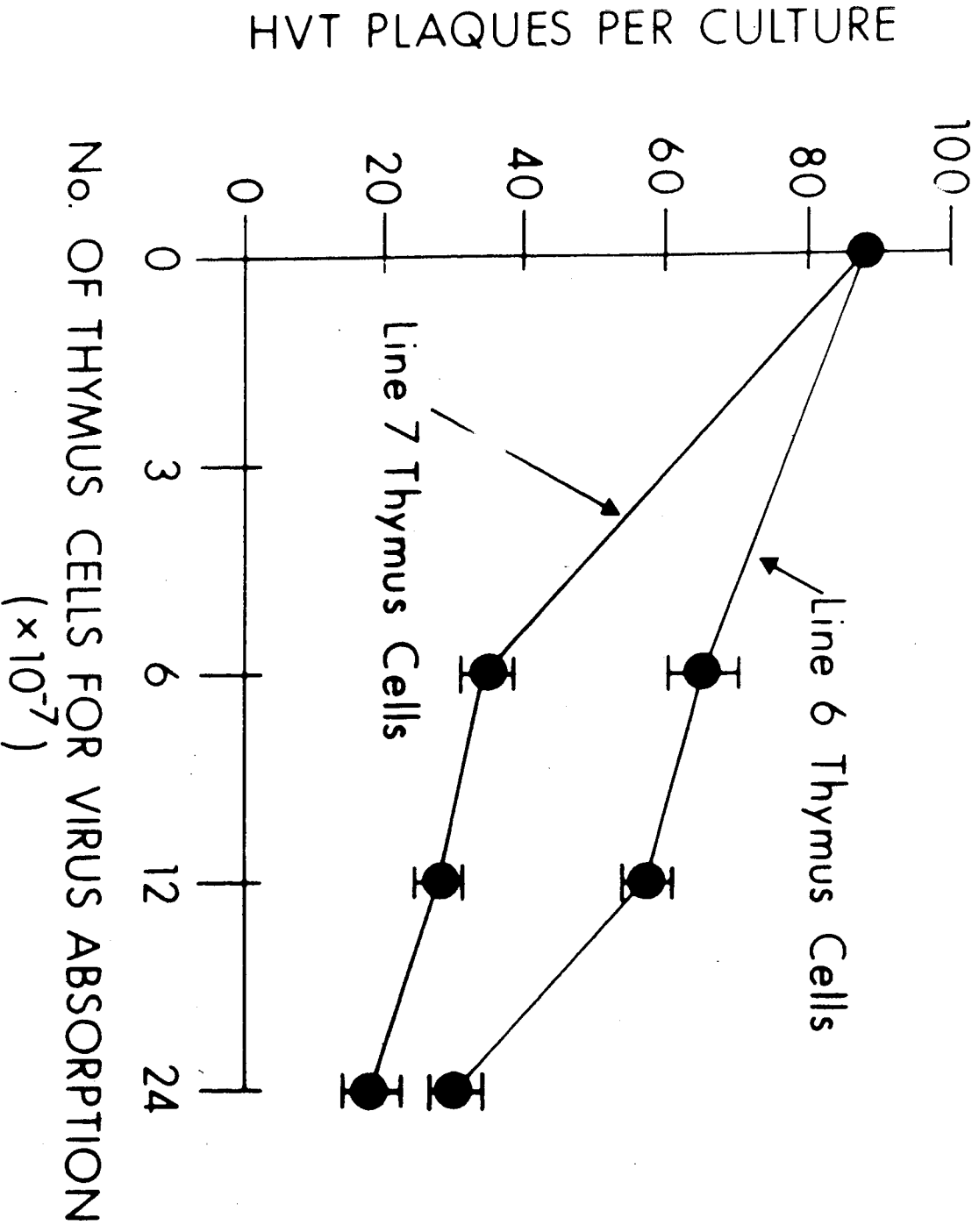
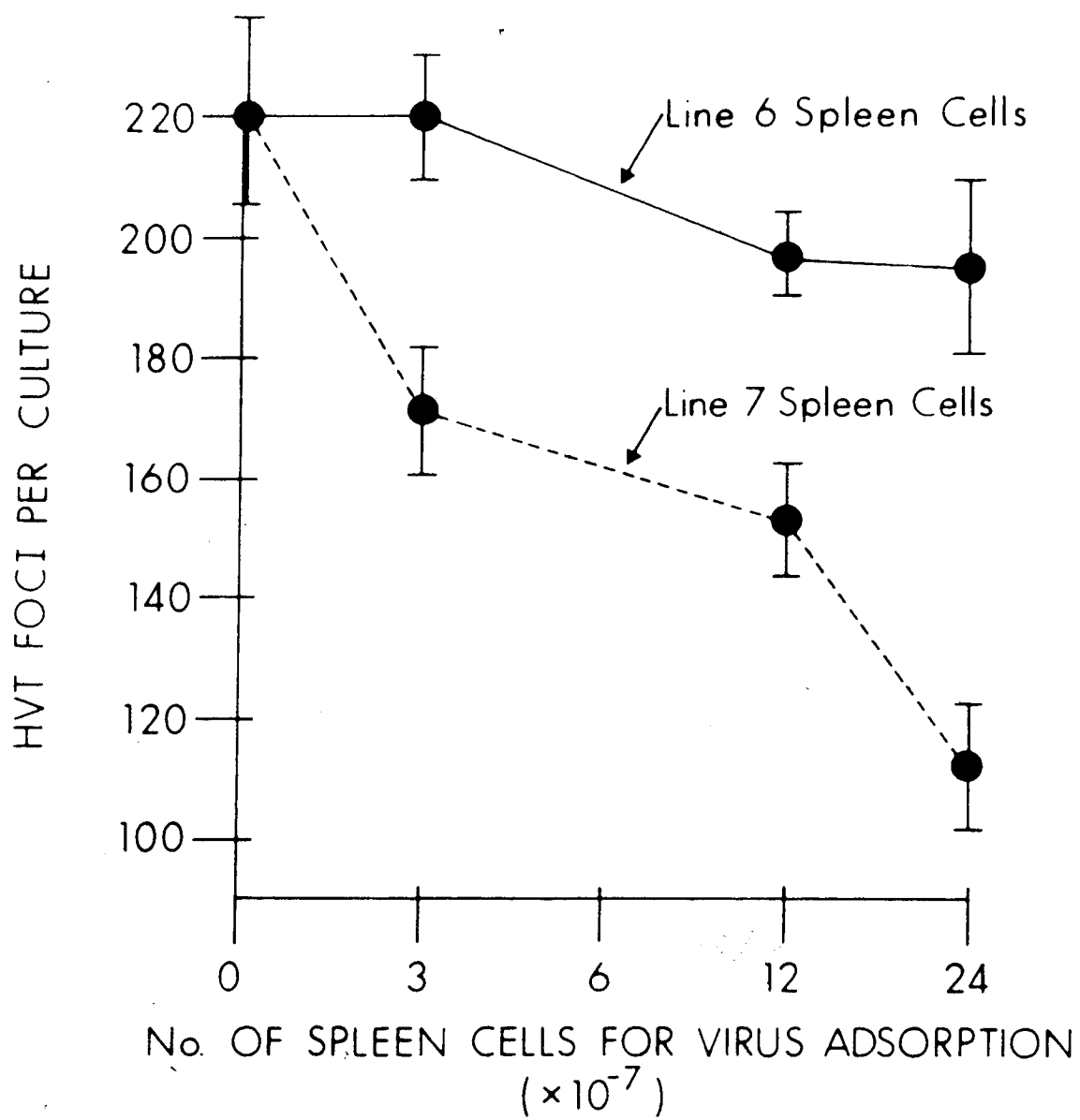


Fig. 3 Adsorption of HVT by line 6 and line 7 spleen cells. HVT was prepared as described in Fig. 1. Cell suspensions from spleens of 3-4 week-old donors were prepared by collagenase digestion, washed three times in medium and mixed with aliquots of HVT. Only those cells having morphologies characteristic of lymphocytes were counted and included in the calculation of spleen cell number for data shown. Virus adsorption was carried out at room temperature for 1 h and the cells were then pelleted by centrifugation. The amount of infectious HVT remaining in the supernatant was estimated by focus formation on replicate cultures of CEF of Hy-line SC origin. Error bars indicate 1 s.d. from the mean.



more infectious centres than did the line-6 spleen cells (Table I).

Since cell free MDV was not available, another approach was taken to corroborate the findings reported above. Line 6 and 7 spleen cells were adoptively transferred into separate groups of irradiated, histocompatible, third party recipients. Recipient chicks were then naturally exposed by contact to virulent MDV. The irradiation protocol used reduced the background of host-specific infectious centres sufficiently so that the infection of donor cells could be measured at seven day days post-infection. The number of infected spleen cells at this time was determined by an in ovo assay (Longenecker et al., 1975). As determined by this procedure four times more line 7 spleen cells than line-6 spleen cells were infected with MDV (Table I).

#### B. Marek's Disease Mortality in Haemopoietic Chimeras

The experiments outlined in the previous section (A) suggested that it might be possible to make a resistant (line 6) bird more susceptible to MD by providing a source of susceptible (line 7) target cells. For this purpose, two sources of line 7 cells were used for transplantation into line 6 recipients. First, classical haemopoietic chimeras were prepared by intravenous injection of line 7 embryonic spleen cells into line 6 embryos.

Four identical experiments were performed and the results were pooled (Table II). Line 6 chicks which had been

Table 1. Infectious centres induced by spleen cells exposed to HVT in vitro or MDV in vivo

Spleen cell Source	HVT lesions per $1 \times 10^7$ cultured spleen cells	MDV lesions per $5 \times 10^7$ injected spleen cells
Line 6	$33.5 \pm 25.5$	$9.4 \pm 6.5$
Line 7	$223.9 \pm 38.0$	$38.6 \pm 3.6$

For determination of infectious centres after in vitro exposure to HVT, aliquots of the spleen cells used for the HVT adsorption studies of Fig. 3 were washed three times in medium and cultured in RPMI-1640 with 10% fetal calf serum for 24h at 37°C at a density of  $5 \times 10^6$  spleen cells per ml. Cells were then washed twice in medium and infectious centres were assayed in ovo. For determination of infectious centres after in vivo exposure to MDV, groups of five Hy-line SC ( $B^2/B^2$ ) third-party chicks were given 500r of gamma-irradiation on days 3 and 7 post-hatching and reconstituted with  $1 \times 10^8$  spleen cells obtained from 6-week-old line-6 or line-7 donors. Following a 7-d period of natural exposure of the recipients to MDV, spleens from each group were pooled and the number of infectious centres was assayed in ovo in  $B^2/B^2$  eggs histocompatible with the donor MHC. Background host-specific infectious centres in unreconstituted, gamma-irradiated, MDV-exposed chicks formed less than 15% of the observed totals and this value was subtracted from the data to obtain donor-specific infectious centres. Error values are 1 standard deviation from the mean.



Table II. Marek's Disease Mortality in Haemopoietic Chimaeras

Experiment No.	Line 7 -> line 6 chimaeras	Line 6 -> line 6 controls
1	6/10	4/10
2	2/9	2/6
3	8/18	3/19
4	8/16	7/17
TOTAL	24/53	16/52
% Mortality	45.3%	30.8%

$\chi^2$  for 1 degree of freedom  $0.2 < p < 0.3$  N.S.

injected as embryos with line 7 embryonic spleen cells and were presumably chimaeric were not significantly more susceptible to MD than control line 6 chicks which had been injected with line 6 embryonic spleen cells. Thus, the mere presence of line 7 haemopoietic cells apparently did not result in an increased mortality due to MD. It is noteworthy that the percentage mortality of the controls is higher than expected for highly resistant line 6 birds. I attribute this to the presence of a high MDV load in our infectious quarters at this time since all of the genetically resistant lines (line 6, line N, strain K) were showing, on the average, a percentage MD mortality of between 15-30% while the susceptible lines (line 7, line P and strain S) were dying at a much higher rate (95 - 100% mortality).

#### C. Marek's Disease Mortality in Chicks Which Received Thymus Transplants

To provide a more direct source of T lymphocytes, the putative target cells for transformation, line 7 thymuses were transplanted into newly hatched, thymectomised line 6 recipients. A comparison of the MD specific mortality in group A (52.8%) and group C (16.7%) in Table III shows that recipients of line 7 thymuses experienced significantly higher mortality than did chicks which received line 6 thymus transplants. We observed no significant differences in MD mortality between thymectomised line 6 birds, sham-thymectomised line 6 birds and thymectomised line 6

Table III. Marek's Disease Mortality in Chickens Which Received Thymus Transplants

GROUP	DEAD/TOTAL	%MORTALITY
A line 7 N --> line 6 TX	38/72	52.8
B line 7 R --> line 6 TX	10/24	41.7
C line 6 N --> line 6 TX	6/36	16.7
D line 6 TX	12/46	26.0
E line 6 sham TX	3/17	17.6

Cumulative MD-specific mortality is shown for a period of 14 weeks of contact exposure to MDV after hatching. N=normal, non-irradiated donor thymus; TX=thymec omised; R=1,000r gamma-irradiated donor thymus. Using Fischer's calculation of exact probability, P=0.00046 for a two-tailed comparison of groups A and C and P=0.033 for a one tailed comparison of groups B and C.

birds which received a line 6 thymus transplant. This finding serves to control for the possibility that the line 7 --> line 6 transplanted birds showed a higher mortality simply because the donor thymus failed to reconstitute a depleted T-cell compartment. In addition, there have been no reports that thymectomy per se has an effect on MD mortality, although neonatal thymectomy combined with irradiation (Sharma et al., 1975) or anti-T cell serum treatment (Shieh and Sevoian, 1976) can affect MD mortality and MDV viraemia.

The finding that transplantation of susceptible thymuses made the resistant birds more susceptible but that the presence of haematopoietic cells from the susceptible line did not, could mean that (1) thymus transplant simply provide more susceptible target cells at the appropriate stage of differentiation than a more primitive population of embryonic spleen cells, or (2) the genotype of the thymic microenvironment may influence the target-cell phenotype, as stem cells can be induced to undergo differentiation into mature T cells in the thymus gland. In support of the latter possibility, we have found that transplantation of irradiated (1,000r) line 7 thymuses also increased the susceptibility of line 6 chicks (Table III).

#### D. Marek's Disease Mortality in Recipients of SPF, Adult Line 7 Spleen Cells

If one postulates that the line 7 thymic microenvironment exerts a modifying influence on the phenotype of MDV target cells, then another prediction can be made. Unlike the adoptive transfer of embryonic spleen cells, adoptive transfer of line 7 adult spleen, a portion of which have presumably migrated through a "susceptible" thymic microenvironment, should be sufficient to increase susceptibility to MD.

Spleen cells from 8 week-old resistant line 6 and susceptible line birds were adoptively transferred into sub-lethally irradiated 8 day-old recipients as described in materials and methods. MD-specific mortality was recorded during a period of fourteen weeks following initiation of contact exposure to seeder birds infected with the BC-1 isolate of MDV (Table IV). Mortality in line 6 recipients of line 7 cells (group B) was significantly increased to 50% from the value of 13.5% observed in line 6 birds reconstituted with line 6 cells (group A;  $p < 0.05$ ). The effect was not simply due to non-specific failure of the line 7 spleen cells to restore the lymphoid compartment in the irradiated recipients as the dose of irradiation in this experiment was sublethal and only 6.3% mortality was observed in the unreconstituted control line 6 birds (group C). At the level of MDV exposure in this experiment the difference in mortality between the unirradiated line 6 and

Table IV. Marek's Disease Mortality in Gamma-Irradiated Recipients Reconstituted With SPF Adult Spleen Cells

	Dead/Total	%Mortality
A Line 6-->Line 6 Irradiated	5/37	13.5
B Line 7-->Line 6 Irradiated	19/38	50
C Line 6 Irradiated, Unreconstituted	2/32	6.3
D Line 6 Normal	0/21	0
E Line 7 Normal	8/8	100
F Line 6-->Line 7 Irradiated	14/15	93

Recipient chicks received 2 x 500r gamma irradiation on days 3 and 8 post-hatching. Following reconstitution with  $2 \times 10^8$  splenic lymphocytes from SPF 8 week-old donors, chicks were contact exposed to MDV for 14 weeks. Mortality was increased significantly in chicks receiving line 7 cells (Group B) over those which received line 6 cells (Group A,  $P < 0.05$ ).

line 7 controls (groups D and E) was maximal. If the resistance of line 6 were to be explained by the presence of an especially effective immune response, one might predict that transfer of line 6 spleen cells into irradiated line 7 chicks would have a protective effect. As shown by the 93% mortality in group F, no significant protection was conferred by this treatment. This result does not however, rule out the possibility that an MD-related immune response difference does exist between line 6 and line 7. Such a difference could be a property which is not readily transferred with spleen cells.

#### E. The Effect of Thymic Microenvironment on the Capacity of Thymic Lymphocytes to Adsorb HVT

The results reported in Table III and Table IV show that susceptibility can be substantially increased in line 6 birds following transplantation of intact or irradiated line 7 thymus as well as by adoptive transfer of adult line 7 spleen cells. The necessary property for eliciting this increase in MD mortality was not present in more primitive population of line 7 embryonic spleen cells (Table II). Based on these facts it was postulated that the line 7 thymic microenvironment influences the target cell phenotype.

To test for this possibility, I first irradiated 4 week-old line 6 and line 7 chickens lethally with 1000r of gamma irradiation. After 4 hours time when the thymuses of

these birds were found to be depleted of detectable lymphoid elements, thymic fragments were grafted onto the CAM's of day 11 resistant line 6 embryos. During the next 7 days the grafts were revascularized and once again became populated with lymphoid cells.

On the eighteenth day of embryonic development the thymus grafts were removed from the CAM, a single cell suspension was prepared, and the ability of the fraction containing the thymic lymphocytes to adsorb infectious HVT was then determined in vitro using the focus inhibition assay (FIA). As shown by Figure 4, thymocytes prepared from the line 7 grafts adsorbed significantly more virus than did the cells obtained from the line 6 grafts. Again, the difference is not large but is highly reproducible as the experiment has been repeated four times with consistent findings. Although care was taken in the preparation of the thymus cell suspension so as to remove the large stromal cells prior to the focus inhibition test, a few contaminating nonlymphoid cells might have remained in the suspension. The low ratio of infectious particles adsorbed to cells tested meant that if present, these stromal cells could have been responsible for the difference observed. To ensure that the results obtained reflected properties residing in the lymphocyte fraction an additional control was carried out wherein the HVT adsorption capacity was determined in ungrafted thymic stromal cells following their in situ irradiation. As can be seen in Figure 5, these large



Fig 4. HVT adsorption by the lymphocyte fraction of cells from CAM thymus grafts on line 6 eggs. Capacity to adsorb cell-free HVT was determined by focus inhibition assay. The origin of the grafts is indicated with arrows. Cells from the grafts on 10 line 6 eggs were pooled and assayed in quadruplicate. Error bars indicate one standard deviation from the mean. Lymphocytes obtained from line 7 grafts adsorbed significantly more virus ( $P < 0.01$ ) than did cells from line 6 grafts at the two highest cell concentrations tested.

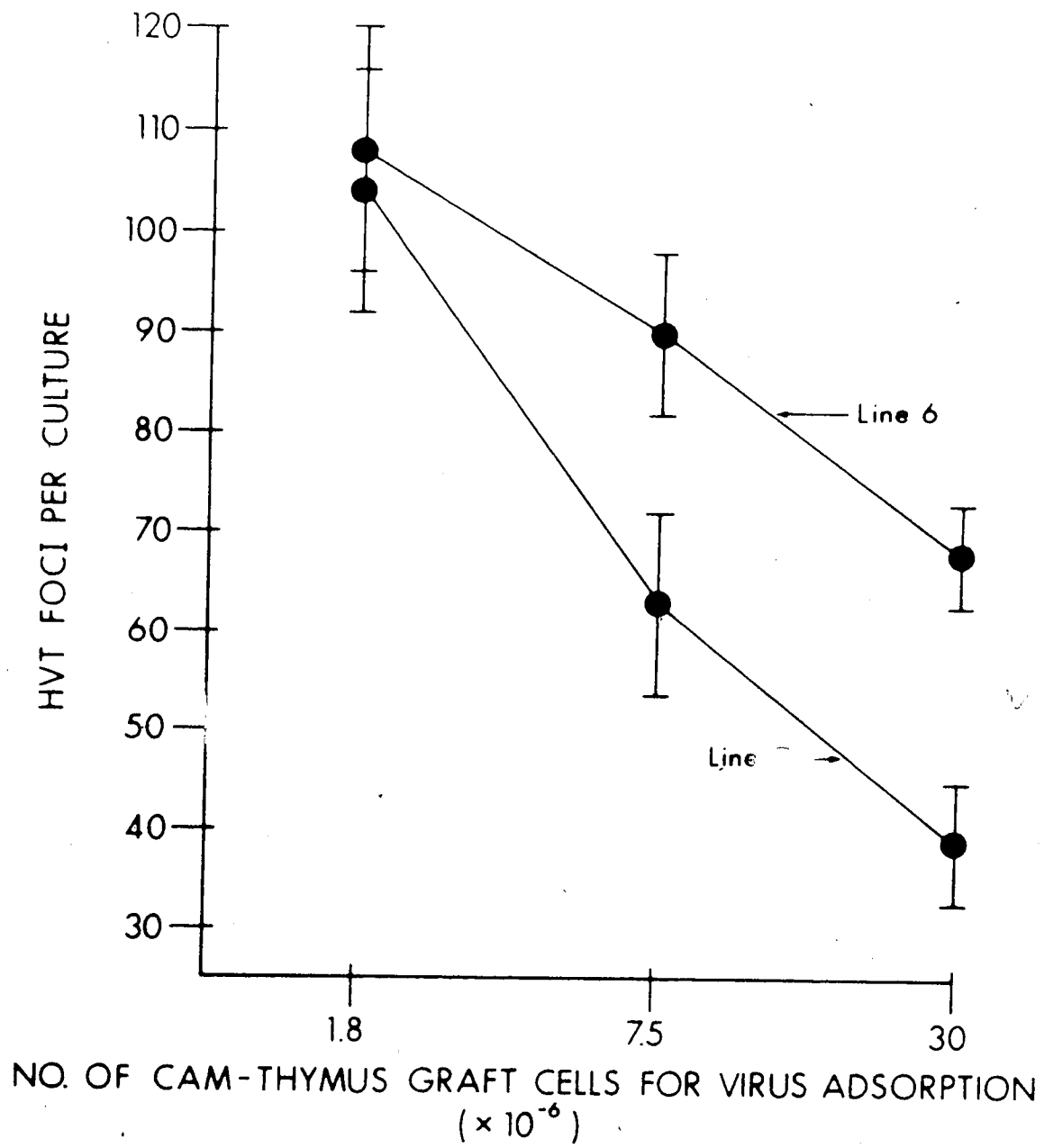
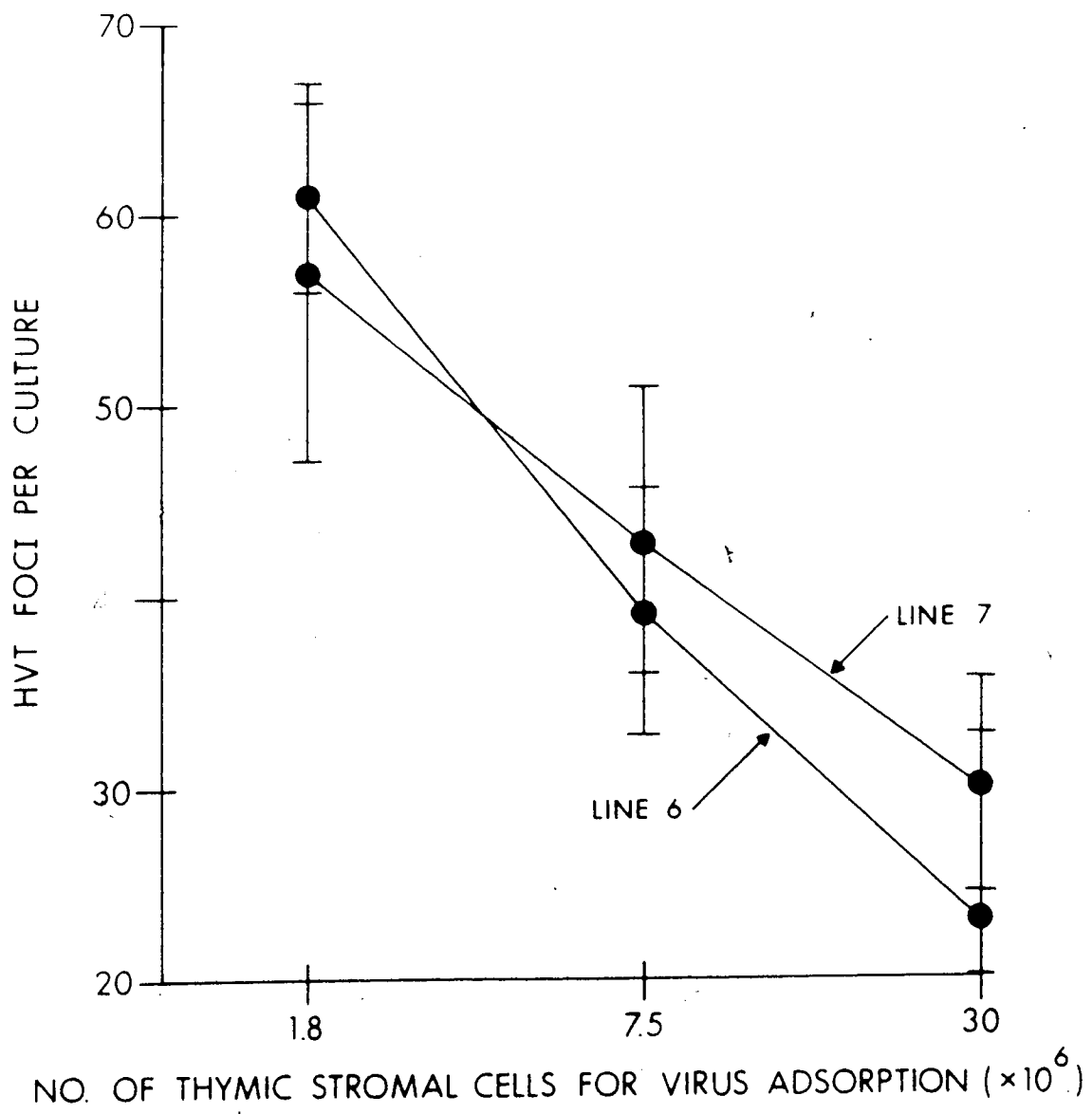


Fig 5. HVT adsorption by thymic stromal cells of line 6 and line 7 origin. 4 hours after in situ irradiation with 1000r from a <sup>137</sup>cesium source, single cell suspensions of thymic stroma were prepared by collagenase digestion followed by velocity sedimentation of the larger stromal cells. The capacity of this population to adsorb cell-free HVT was then determined by focus inhibition assay. Cells from 4 birds of each genotype were tested separately with each test performed in quadruplicate. Data for each point were pooled for the calculation of standard deviations. No significant difference in virus adsorption capacity was observed between stromal cells of line 6 and line 7 origin.



cells did adsorb virus in a dose dependent fashion even slightly more efficiently on a population basis than did the lymphocytes. The important point, nonetheless, is that there was no difference between thymic stromal cells from these two lines with respect to the parameter of virus adsorption.

#### F. Production and Characterization of Monoclonal Antibodies to HVT

##### Introduction

The differential virus adsorption capacities observed in the studies reported here might reflect line-specific differences in the frequencies of virus binding leukocytes. Since the focus inhibition assay measures relative virus adsorption abilities of whole populations of cells and provides no information as to the frequencies of HVT binding leukocytes in line 6 and line 7, another assay system was developed to obtain frequency estimates. In a modification of the antibody directed rosette assay (ADRA) described by Mosmann et al., (1980), attempts were made to use hyperimmune chicken antisera against HVT to measure the binding of HVT to individual cells. First, HVT adsorption to line 6 and line 7 cells was carried out as in previous experiments. After washing the cells to remove unbound virus, CRBC coated with chicken anti-HVT serum were added in hopes that rosettes would form around virus-binding cells. Unfortunately, the nonspecific binding of such coupled CRBC to control cells which had not been incubated with virus was

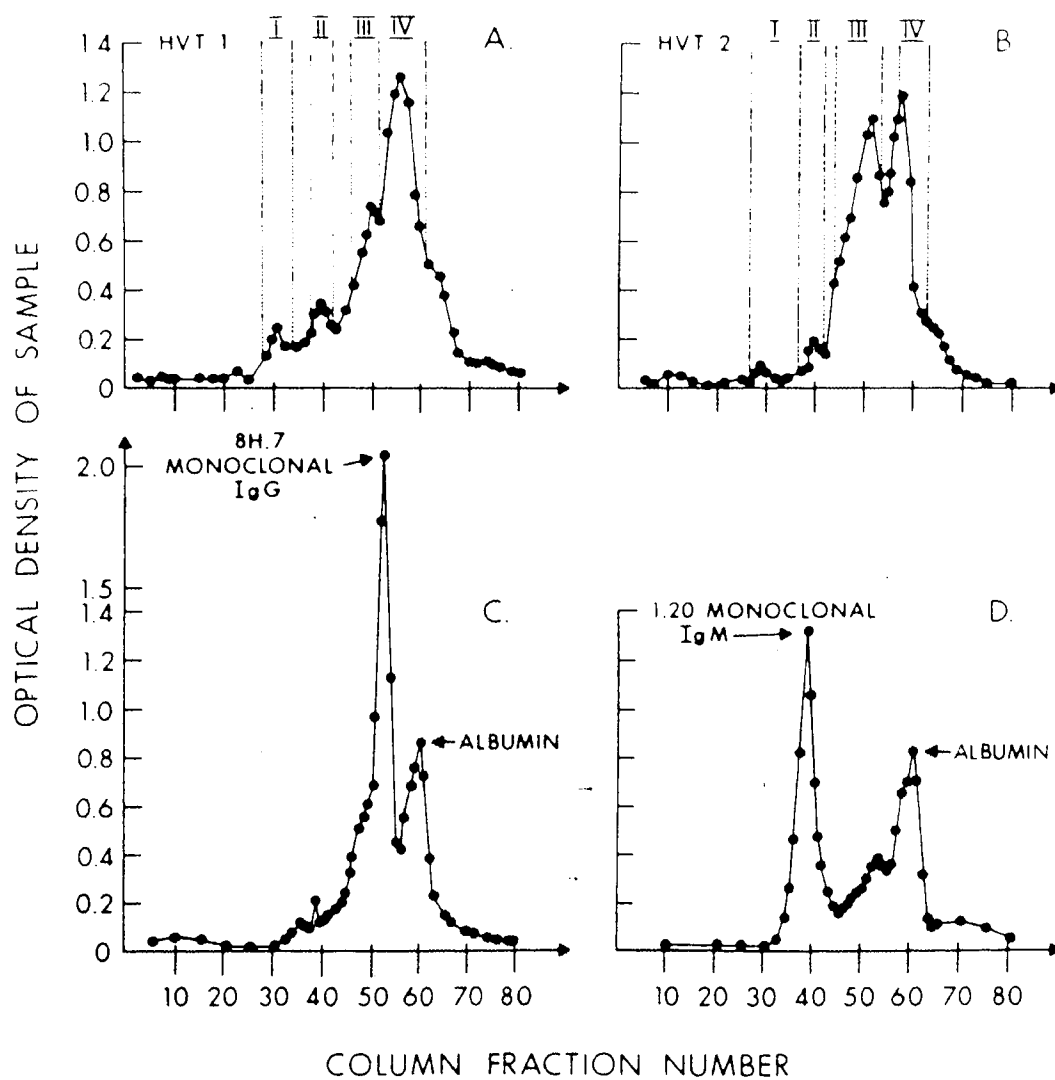
unacceptably high. In other experiments unrelated to this project high background binding of chicken immunoglobulin was also observed in cellular radioimmunoassays (B.M. Longenecker, unpublished data). In order to circumvent this problem, monoclonal (hybridoma) antibodies against HVT were produced as described in materials and methods.

#### Sephacryl-300 Purification of Anti-HVT Monoclonal Antibodies

Out of approximately 1300 hybridoma clones screened, nine produced antibodies which reacted positively against HVT antigens. Of these nine clones, all nine produced antibodies which specifically agglutinated HVT-coupled CRBC and seven synthesized antibodies which bound specifically to HVT antigens as measured by ELISA. Two clones, HVT.1 and HVT.2, which reacted most strongly in both assays were selected for further characterization. First, the clones were adapted for growth as ascites as outlined in (materials and methods). Ascites fluids were then partially purified by Sephacryl-300 gel filtration (Figures 6a-b).

Chromatographic data for known IgM and IgG monoclonal antibodies obtained from the same column is included in Figures 6c and 6d for comparison. Monoclonal antibody 8H.7 (Figure 6c) is of the IgG class and detects an antigen on human lymphocytes (MacLean, et al. 1981). Monoclonal antibody 1.20 (Figure 6d) is of the IgM class and detects an antigen on Marek's disease lymphoblastoid cell lines which is associated with liver specific metastasis (Shearman et al., (1980). Following gel filtration HVT.1 and HVT.2 eluates

Fig 6. Sephacryl-300 gel filtration of hybridoma ascites fluids. Column chromatography was performed as described in materials and methods. The peaks containing serum albumin and known IgM and IgG hybridoma antibodies are indicated with arrows. 6a=HVT.1; 6b=HVT.2; 6c=8H.7, a known IgG hybridoma antibody which detects antigens on human lymphocytes; 6d=1.20, a known IgM monoclonal antibody which detects antigens on MD LCL associated with liver-specific metastasis (Shearman et al., 1980). Fractions from HVT.1 and HVT.2 were pooled as indicated by Roman numerals. Each pool was adjusted to 2.5mg./ml. protein concentration with RPMI 1640 and tested for anti-HVT activity by ELISA, agglutination of HVT-coated CRBC, and virus neutralization.



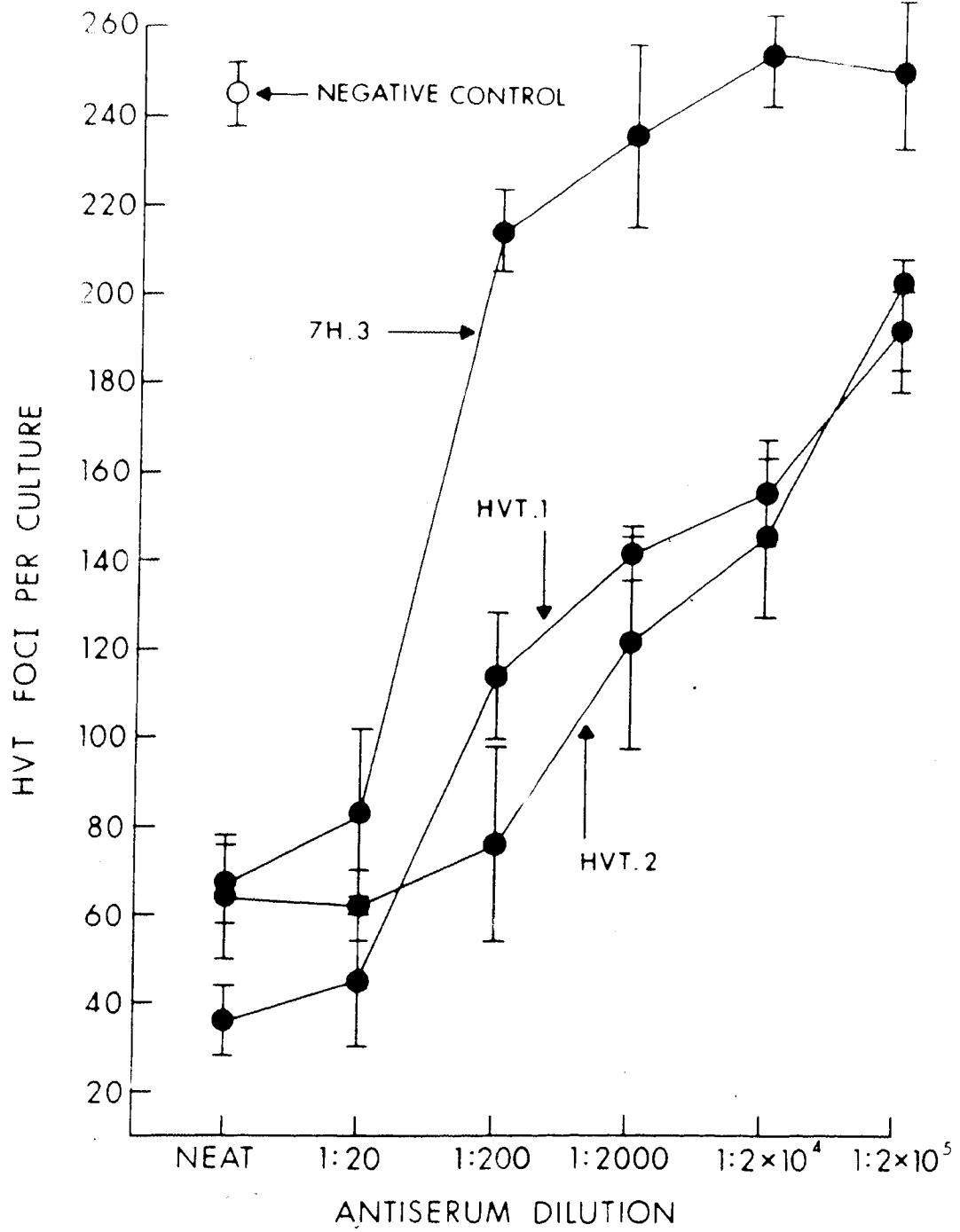


were pooled as indicated by the Roman numerals and again tested for anti-HVT activity by both ELISA and haemagglutination methods. In each case significant reactivity was found only in pool III. By comparison of elution volumes, pool III corresponded to the major, IgG, peak observed for 8H.7 antibody and could be easily distinguished from the IgM antibody, 1.20, also, in comparison to 1.20 ascites fluid, both HVT.1 and HVT.2 ascites fluids showed elevated protein concentrations in the fractions which correspond by position to IgG antibody. On the basis of these observations both HVT.1 and HVT.2 were considered to be of the IgG class.

#### Neutralization of HVT by HVT.1 and HVT.2 Monoclonal Antibodies

The initial screening for anti-HVT antibodies was carried out against membrane fractions from HVT-infected CEF (materials and methods). In order to determine if the antigens detected by HVT.1 and HVT.2 were found on the HVT virion itself, these antibodies were tested in ascites form for virus neutralization (Figure 7). 7H.3 ascitic fluid was included as a suitable negative control since this antibody was of the same, IgG, class as HVT.1 and HVT.1. At the two lowest dilutions all three ascites fluids neutralized cell-free HVT. Neutralization by 7H.3 ascites fluid was not unexpected. A similar effect had been observed with normal serum from one Balb/c mouse in an earlier, abortive attempt at production of anti-HVT hybridomas. High titres of

Fig 7. Neutralization of cell-free HVT by hybridoma ascites fluids. This assay was performed as described in materials and methods. The negative control was HVT plus medium with 0.5% FBS. 7H.3 is an IgG monoclonal antibody which detects antigens on human lymphocytes and serves here as a negative control. Error bars indicate one standard deviation from the mean.



"natural" antibodies to chicken MHC antigens are present in normal mouse sera (Longenecker et al., 1980) and ascites fluids (unpublished observations). "Natural" antibodies of this sort are likely responsible for the HVT neutralization we observed here. Whatever the mechanism, the capacity of 7H.3 antibody to neutralize HVT was rapidly lost with dilution; a significant break in the curve occurs between the 1:20 and 1:200 dilutions HVT.1 and HVT.2, on the other hand, exhibited significant neutralization at all the concentrations tested; even at dilutions on the order of  $1:2 \times 10^5$ . To confirm these results additional virus neutralization tests were carried out with pool III of column purified ascites fluids. Again both HVT.1 and HVT.2 neutralized HVT (Figure 8).

#### Detection of HVT Antigens on the Surface of HVT-Infected CEF

To further define the nature of the antigens detected by HVT.1 and HVT.2, HVT-infected CEF were examined by the following immunofluorescence technique. First coverslip cultures of HVT-infected CEF were fixed with glutaraldehyde at 120 hours post-infection. The coverslips were then incubated with 7H.3, HVT.1 or HVT.2-coupled, fluorescinated latex beads as described in materials and methods. Paired phase contrast and ultraviolet photomicrographs from these studies are presented in Figures 9a-15b. Large numbers of HVT.1-coupled beads bound to the morphologically distinct, rounded cells seen in HVT foci (Figure 9a-b) but not to morphologically normal area adjacent to foci so stained

Fig 8. Neutralization of cell-free HVT by Sephacryl-300 purified hybridoma antibodies. This assay was performed as described in materials and methods. Fractions from column eluates were pooled as indicated in Figure 6 and adjusted to 2.5mg./ml. protein concentration with RPMI 1640 before use. Virus neutralization with pool III is indicated here. Error bars indicate one standard deviation from the mean.

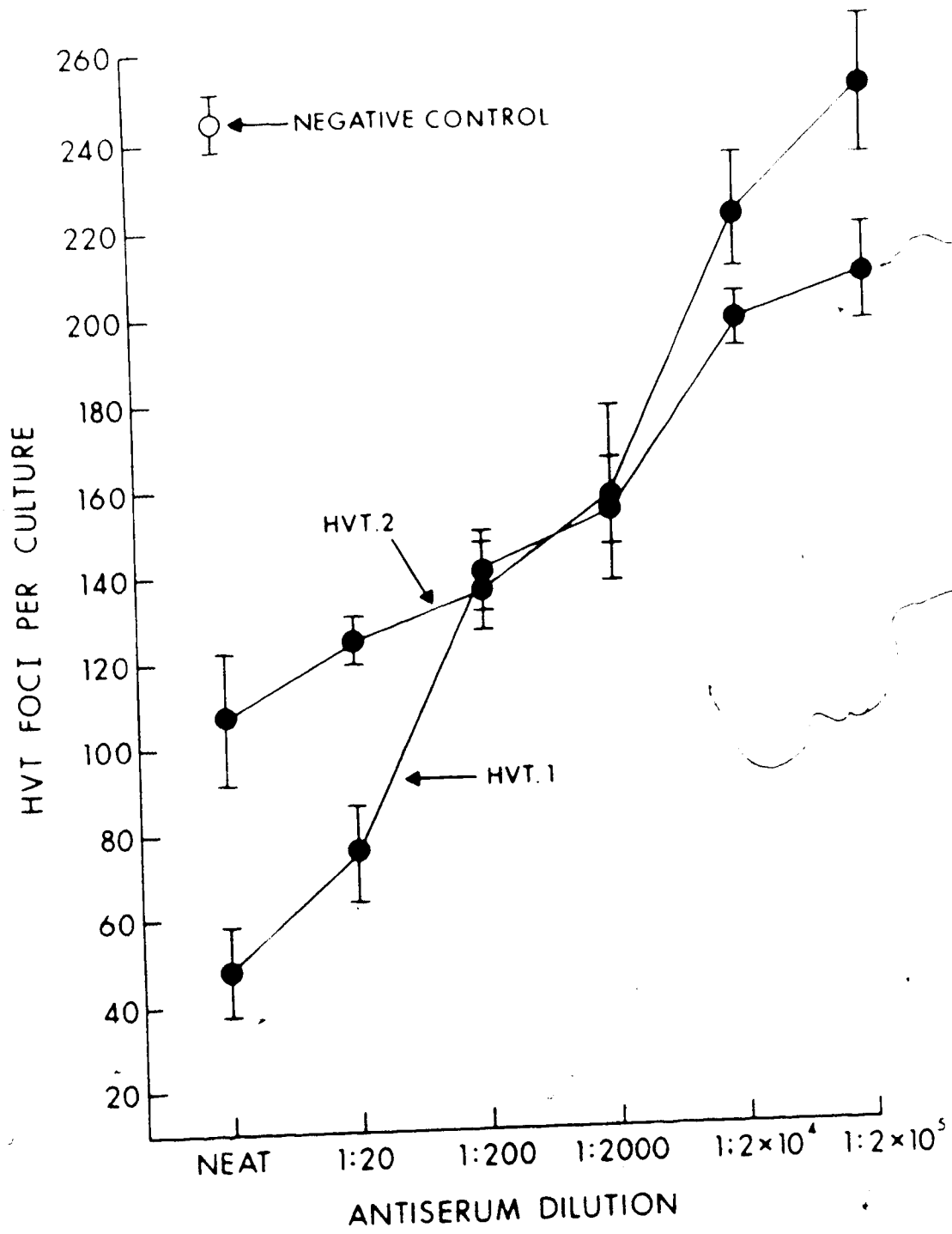


Fig 9. Binding of fluorescinated latex beads coated with hybridoma antibodies to HVT-infected CEF. CEF were grown on glass coverslips in MEM with 0.5% FBS. 120 hours after HVT infection CEF were fixed by a 30 second treatment with 0.1% glutaraldehyde at room temperature. Coverslips were incubated overnight at 4°C with fluorescinated latex beads coated with either HVT.1, HVT.2 or 7H.3 monoclonal antibody which detects antigens on human lymphocyte. After extensive washing with PBS, coverslips were mounted on slides with 1:1 glycerol PBS and examined with a Zeiss fluorescence microscope at a magnification of 200x. Paired photographs of identical culture areas are presented as (a) phase contrast and (b) ultraviolet images. Description of individual pictures is as follows:

Figure	Description
9	HVT focus plus HVT.1 coated beads
10	HVT focus edge plus HVT.1 coated beads
11	Background binding of HVT.1-coated beads to a morphologically normal area of an infected culture
12	HVT focus plus HVT.2-coated beads
13	HVT focus edge plus HVT.2-coated beads
14	Background binding of HVT.1-coated beads to a morphologically normal area of an infected culture
15	HVT focus plus 7H.3-coated beads

Figure 9a.

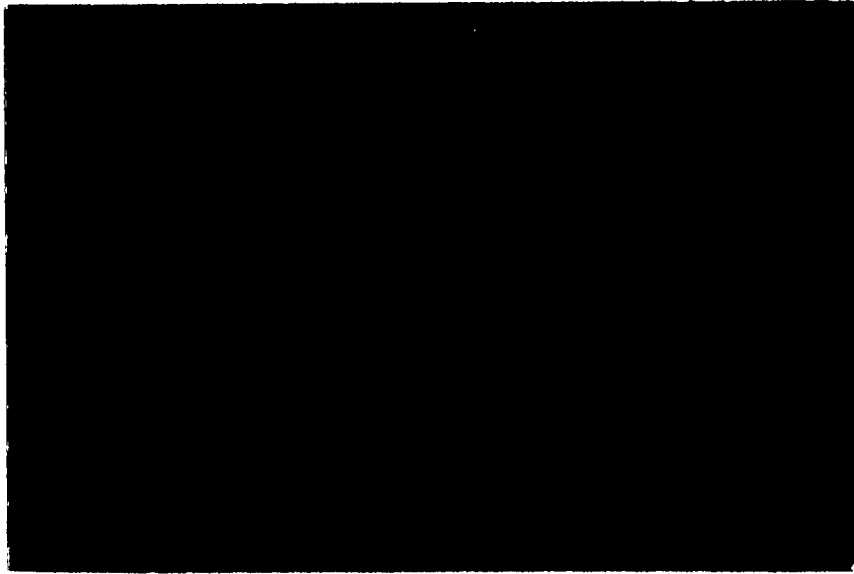


Figure 9b.



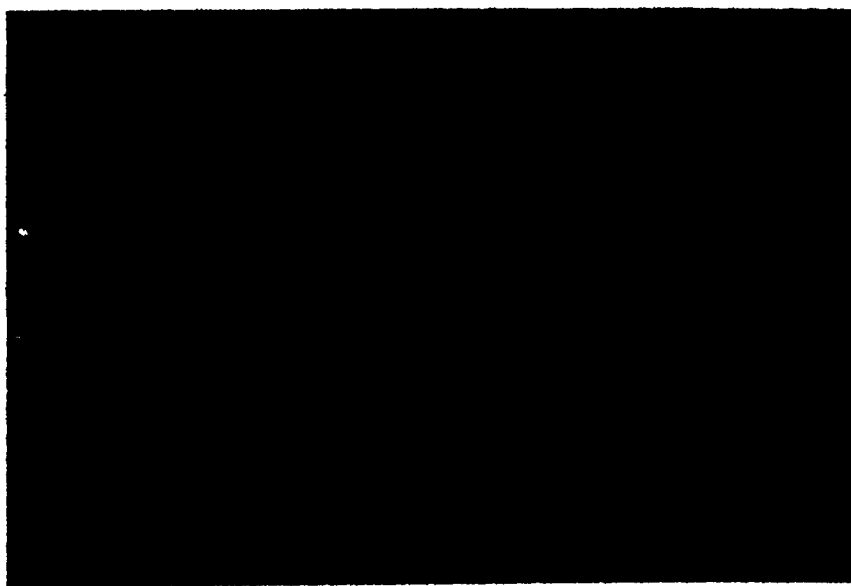
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PAPIER DE COULEUR



Figure 10a.



Figure 10b.

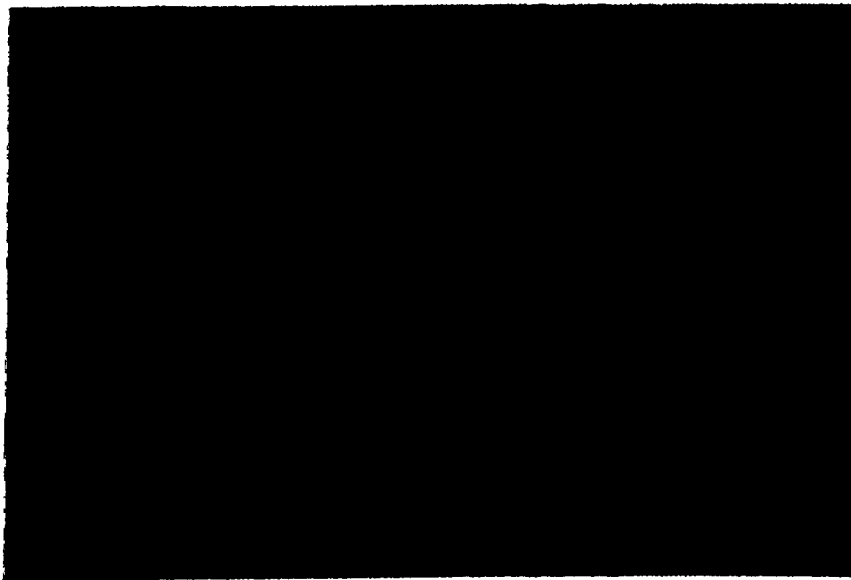


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Figure 11a.



Figure 11b.

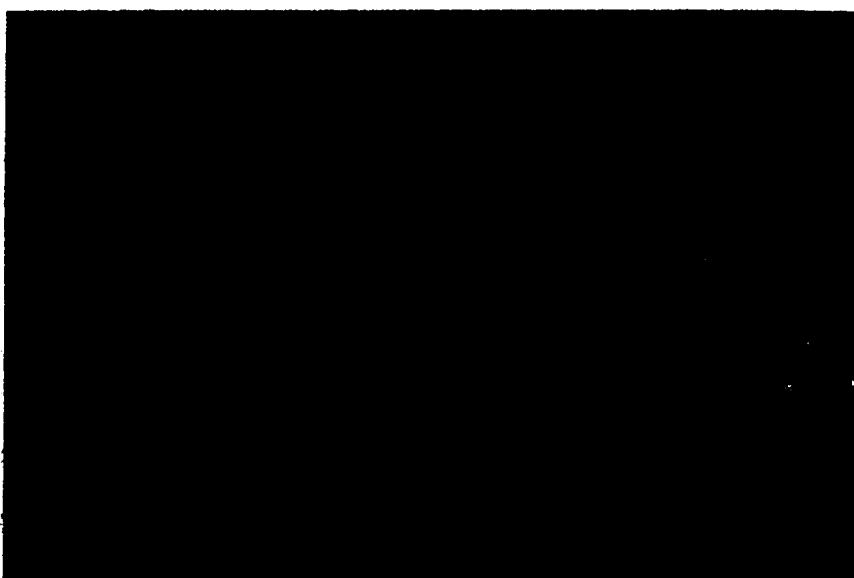


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Figure 12a.



Figure 12b.



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Figure 13a.

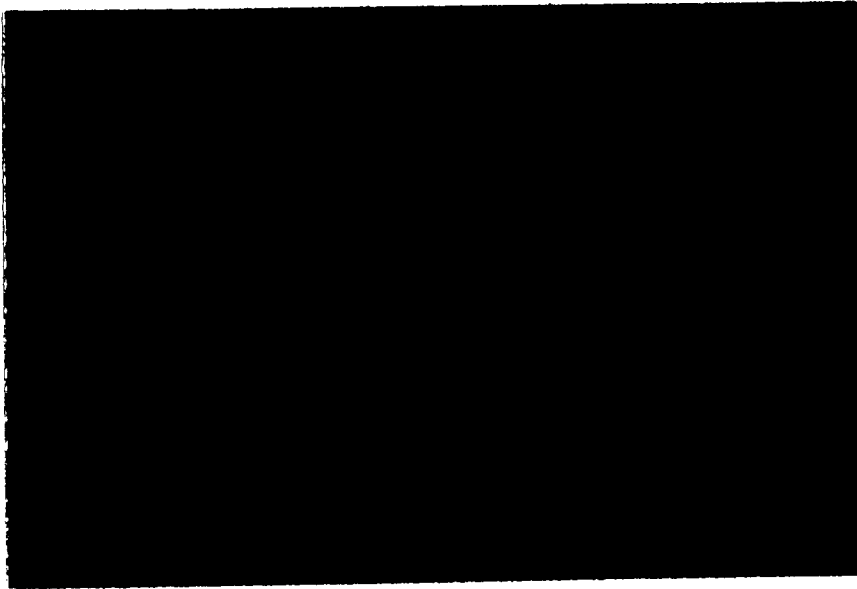
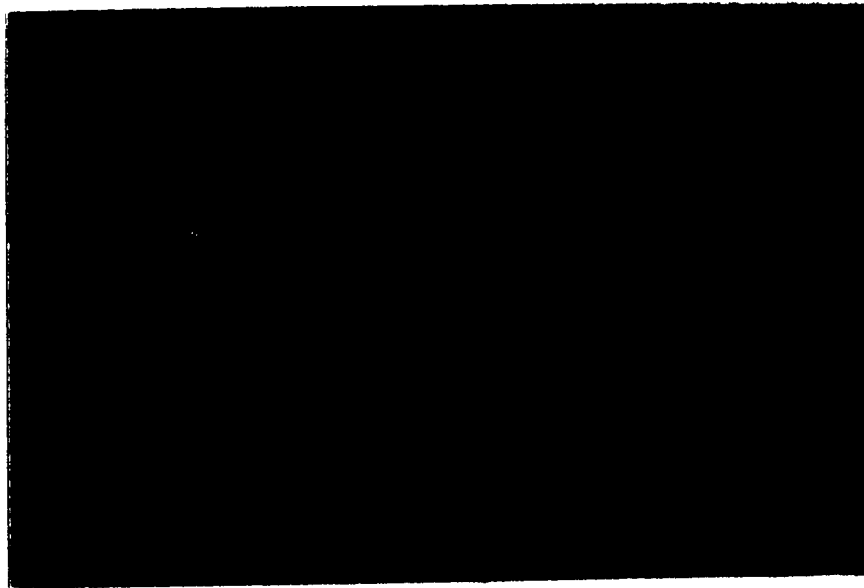


Figure 13b.



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Figure 14a.

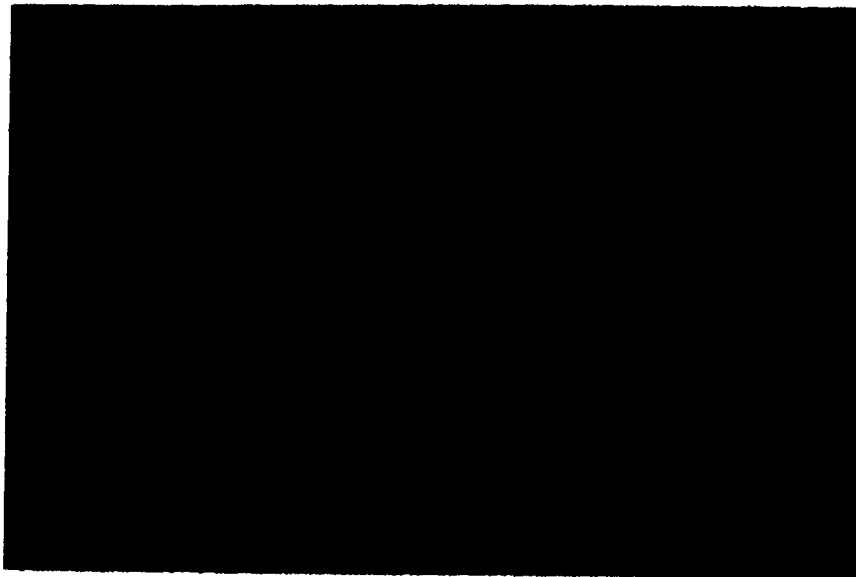


Figure 14b.



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Figure 15a.

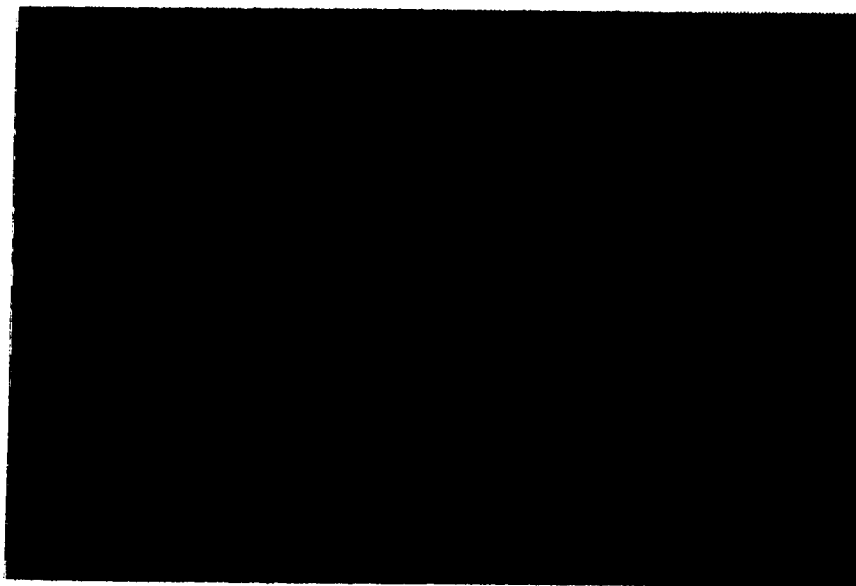
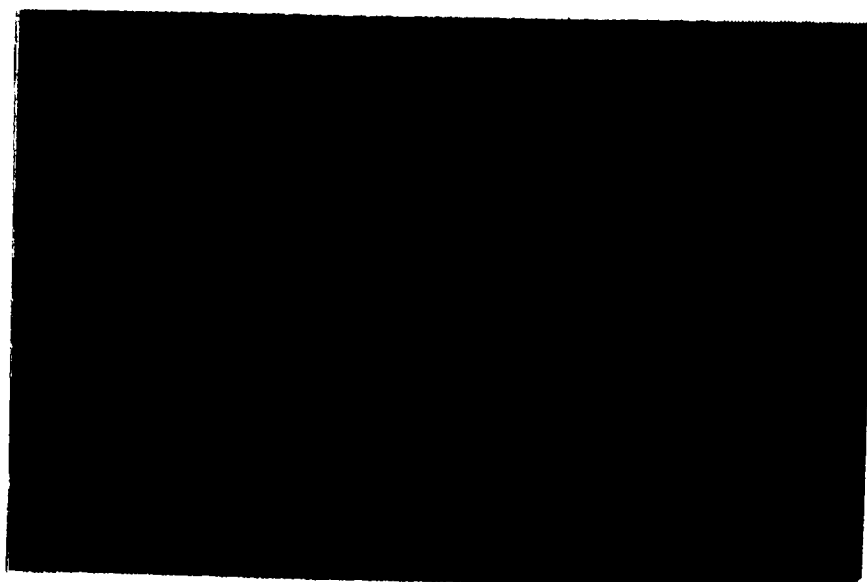


Figure 15b.



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(Figure 10a-b) or to unaffected areas within the same culture (Figure 11a-b). Identical results were obtained with HVT.2-coated beads (Figures 12a-b, HVT focus; 13a-b, area adjacent to focus; 14a-b, unaffected area). 7H.3-coupled beads, which served as a negative control, did not bind significantly to any area of HVT-infected cultures, including HVT foci (Figure 15a-b). Thus, it was clear that the antigenic determinants defined by HVT.1 and HVT.2 were also represented on the surface of infected CEF.

#### G. Frequency of HVT-Binding Cells Measured by ADRA

Having acquired anti-HVT reagents of the necessary specificity and titre, a determination of the frequency of HVT-binding cells in line 6 and line 7 was possible. For this purpose, HVT.2 was employed in the aforementioned antibody-directed rosette assay (ADRA) as described in materials and methods. The data in Table V. indicate that there are significantly higher numbers of such cells in the susceptible line 7. Again, in both line 6 and line 7 the frequency of positive cells is higher in spleen and peripheral blood than in thymus. While large differences were not observed between line 6 and line 7 thymus cells, line specific variations of approximately 20-fold in rosetting cells were recorded in the other two cell sources. Given the comparatively low frequency of positive cells in the thymus (Table V) and the background of the assay it is not surprising that large line specific differences were not obtained in this organ with

Table V. ADRA test for Virus-Binding Cells

Cell Source	Rosettes/10 <sup>3</sup> Leukocytes ( $\pm$ S.D.) <sup>1</sup>		Ratio $\frac{\text{Line 7}}{\text{Line 6}}$
	Line 6(R)	Line 7(S)	
(With Virus)			
Thymus	0.2 $\pm$ 0.1	1.0 $\pm$ 0.5	5.0
Spleen	1.8 $\pm$ 1.5	41.3 $\pm$ 19.5	22.9
Peripheral	2.2 $\pm$ 3.2	46.0 $\pm$ 9.3	20.9
(Without Virus)			
Thymus	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	
Spleen	0.3 $\pm$ 0.2	0.3 $\pm$ 0.3	
Peripheral Blood	0.1 $\pm$ 0.4	0.5 $\pm$ 1.2	

<sup>1</sup>ISOLYMPH Purified single cell suspensions of thymus, spleen and peripheral blood leukocytes were assayed for the frequency of HVT binding cells by ADRA. 4 individual 6 week-old SPF birds of each genotype were tested separately. Each organ was assayed in triplicate and data were pooled to calculate standard deviations from the mean. Spleen and peripheral blood leukocytes from line 7 displayed a significantly higher frequency of virus binding cells than did corresponding cell populations in line 6.



these tests.

#### H. MDV Infectious Centres in Spleen and Peripheral Blood of Recipients of Line 6 and Line 7 Spleen Cells

The line-specific difference in overall virus-binding capacity which was apparent in initial experiments was also reflected by the frequency of HVT-binding cells. Given this fact, it was important to confirm and extend the early observation that HVT adsorption phenotype correlated with a higher frequency of MDV infected cells in vitro. In the first experiment, irradiated, histocompatible, third-party, Hy-Line SC chicks were reconstituted with unprimed, SPF, spleen cells from line 6 and 7 donors and contact exposed to MDV immediately. Thirteen days later the recipients' spleens were removed and infectious centres in the leukocyte fraction were assayed in ovo in Hy-Line SC embryos as described previously (Longenecker et al., 1975, Figure 16). At the highest dose of injected cells it became difficult to estimate accurately the number of viral lesions on the CAM and the sensitivity of the assay was limiting at this point (Figure 17). In confirmation of the earlier data (Table I), at lower numbers of injected cells 3.5 to 7.0 times more infected cells were found in the recipients of line 7 spleen cells compared to recipients of line 6 spleen cells. At this time point the background number of infected cells in the unreconstituted control was negligible. The frequencies of infected cells/injected cells at the midpoint of the graph

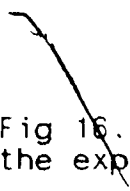
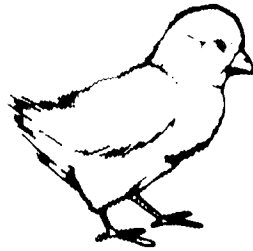


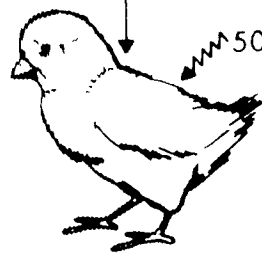
Fig 16. Schematic representation of the protocol used for the experiment presented in Figure 17.

PROTOCOL FOR GRAPH



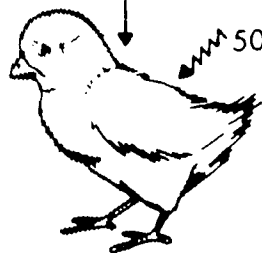
HY-LINE SC ( $B^2/B^2$ ) CHICKS

HATCH



500R  $\gamma$ -IRRADIATION

DAY 3



500R  $\gamma$ -IRRADIATION

DAY 8

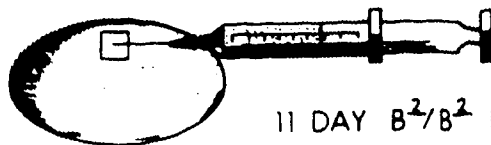
RECONSTITUTE WITH  $10^8$  SPLEEN CELLS FROM 6-WEEK OLD  
SPF LINE 6 (R) OR LINE 7 (S) HISTOCOMPATIBLE DONORS

NATURAL EXPOSURE TO MDV

DAY 21

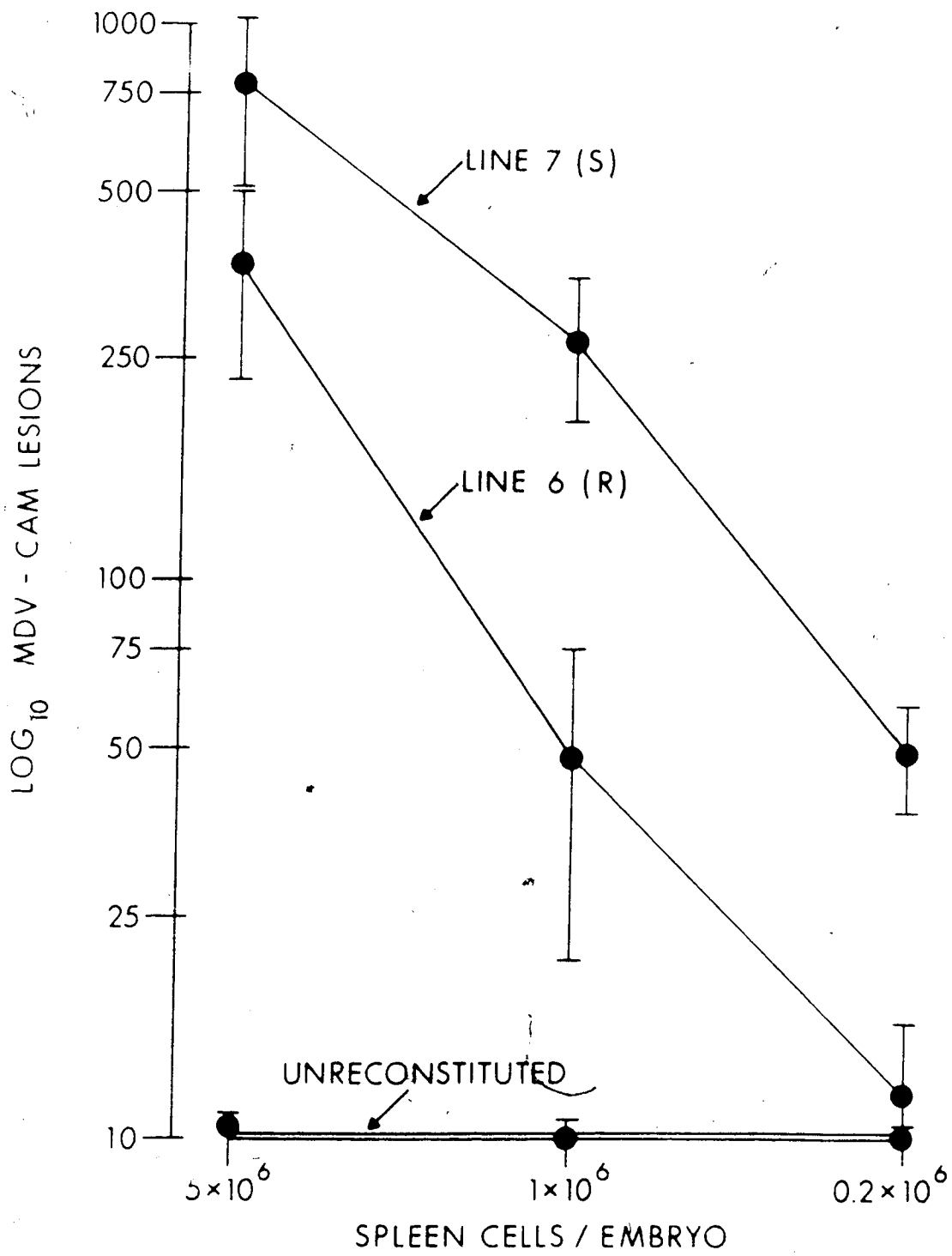
COLLAGENASE SUSPENSION OF RECIPIENT SPLEEN CELLS

IN OVO ASSAY FOR INFECTIOUS CENTERS



11 DAY  $B^2/B^2$  EMBRYOS

Fig 17. MDV infectious centres in spleens of third-party recipients of line 6 and line 7 SPF spleen cells. Hy-Line SC B<sup>2</sup>/B<sup>2</sup> chicks received 2x500r of gamma irradiation on days 3 and 8 posthatching. After adoptive transfer of 2x10<sup>8</sup> splenic lymphocytes from SPF line 6 and line 7 donors birds were contact exposed to MDV. The number of MDV infectious centres associated with splenic lymphocytes was determined 13 days later by in ovo assay (Longenecker et al., 1977). 5 birds were tested per group with 10 replicate tests per bird. Error bars indicate ± 1 standard deviation from the mean.

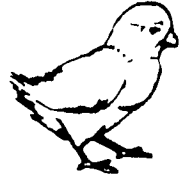


were 1 in 3,030 for line 7 cell recipients and 1 in 20,800 for the line 6 groups. These are probably underestimates since the assay used measures only relative differences in numbers of infected centres rather than their total number.

Since it has been shown in a number of studies that the titre of cell-associated MDV in the lymphoid organs behaves in a cyclical manner (reviewed by Payne *et al.*, 1976), an additional time course study was undertaken in similarly prepared irradiation chimeras. In these tests the recipients were contact exposed to MDV for 12 days prior to their reconstitution with SPF line 6 and line 7 spleen cells. Peripheral blood was assayed sequentially for infectious centres using the *in ovo* assay (Figure 18). The results (Figure 19) indicated that the observed difference between the recipients of line 6 and line 7 spleen cells was time dependent. In this case no appreciable difference in titre between the groups was observed at the earliest time points. At 17 days post-reconstitution a large and highly significant divergence in the MDV titres was recorded. In agreement with data from similar tests done with normal line 6 and line 7 chickens (Fredericksen *et al.*, 1977), the number of MDV infected centres in the line 7 groups declined sharply after the initial peak.

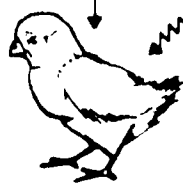
Fig 18. Schematic representation of the protocol used for the experiments presented in Figure 19.

PROTOCOL FOR GRAPH



HY-LINE SC ( $B^2/B^2$ ) CHICKS

HATCH



500R  $\gamma$  - IRRADIATION

DAY 3



500R  $\gamma$  - IRRADIATION

DAY 8

NATURAL EXPOSURE TO MDV

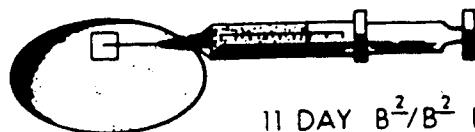


DAY 20

RECONSTITUTE WITH  $10^8$  SPLEEN CELLS FROM 6-WEEK OLD  
SPF LINE 6 (R) OR LINE 7 (S) HISTOCOMPATIBLE DONORS

SAMPLE PERIPHERAL BLOOD FROM RECIPIENTS

IN OVO ASSAY FOR INFECTIOUS CENTERS

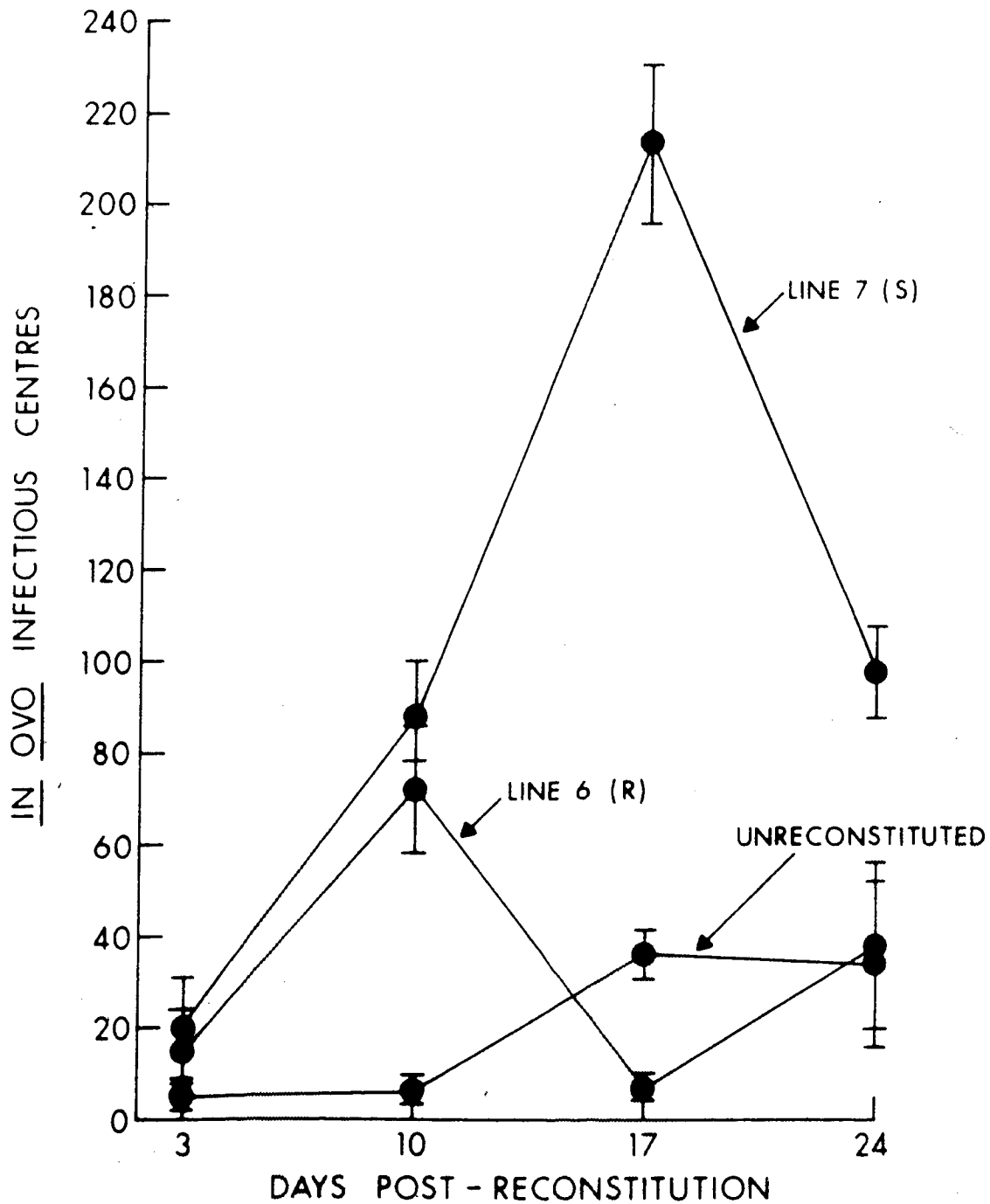


11 DAY  $B^2/B^2$  EMBRYOS



Fig 19. Time course study of MDV infectious centres in peripheral blood of irradiation chimeras. Hy-Line SC B<sup>2</sup>/B<sup>2</sup> chicks received 2x500r of gamma irradiation on days 3 and 8 post-hatching. After 12 days of contact exposure to MDV chicks received 10<sup>8</sup> splenic lymphocytes from SPF line 6 and line 7 donors. The number of MDV infectious centres in peripheral blood was determined by in ovo assay (Longenecker et al., 1975). Each test embryo received 0.1ml of a 1:5 dilution of citrated whole blood. 5 chicks were tested for each time point with 10 replicate tests per chick. Error bars indicate  $\pm 1$  standard deviation from the mean.

TIME COURSE STUDY OF MDV INFECTION CENTRES  
IN IRRADIATION CHIMERAS



## V. Discussion

### A. HVT Infection In Vitro

The early experiments of this project revealed that line 6 and line 7 cells differ significantly in their capacity to adsorb HVT (Figures 2 and 3). Although the magnitude of the observed difference was never large, it was consistent through six experiments. Populations of spleen and thymus cells from line 7 adsorbed approximately twice as much HVT as did comparable line 6 cells. It would, indeed, be foolish to arbitrarily select a numerical standard by which such line-specific differences would acquire biological significance. First of all, in avian RNA tumour virus systems the relative number of virus "hits" required for in vitro transformation can be determined. In some cases, only small differences exist between resistant and susceptible cells (reviewed by Weiss, 1975). No such information exists about in vitro MDV-induced transformation. Second, one has no way of knowing how finely balanced the processes of transformation and immune surveillance are in vivo.

When spleen cells used in adsorption studies were cultured overnight and then assayed in ovo for infected cells, over four times more infected centres were induced by line 7 cells than by line 6 cells (Table I). Thus, measurements of the initial event in infection, adsorption, and the final rescue of infectious virus were directly

correlated. That other cell types from these lines do behave identically in response to herpesvirus infection was evident since CEF from both lines replicated HVT equally well in vitro (Figure 1).

The object of the experiments outlined above was simply to establish the general precedent that cells from the lymphoid organs of lines 6 and 7 do not necessarily respond identically to herpesvirus infection. Since MDV and HVT are different viruses, one cannot make a direct inference about infection of line 6 and 7 cells with MDV. The cell-associated character of MDV ruled out in vitro investigations of the sort carried out with HVT. Line 6 and 7 cells were, therefore, exposed to MDV in vivo.

#### B. Mortality Experiments

In mortality experiments MD susceptibility was increased in 11 line 6 birds following transplantation of normal or irradiated line 7 thymuses, (Table III) but not following transfer of more primitive embryonic line 7 lymphocyte precursors (Table II). Again, it should be mentioned that, due to a high level of exposure to MDV, tumour incidence in line 6 control birds was abnormally high in the experiment in which embryonic spleen cells were transferred. Consistent with the thymus transplant experiments are the results where similar increases in MD mortality were seen after transfer of adult, SPF line 7 spleen cells into line 6 recipients. Alloantisera against

the Ly-4 antigens were obtained from Dr. T. Fredericksen in hopes that they would be useful in determining the degree of T-lymphocyte chimerism in the chicks used for the mortality tests. Unfortunately, these antisera were of low titre and could not be diluted more than 1:16 before they became ineffective in sensitive cellular radioimmunoassays. These anti-Ly-4 sera are effective for typing homogeneous lymphocyte populations in breeding experiments but they were useless for the detection of even high levels of lymphocytic chimerism. Even though the degree of chimerism could not be determined, these experiments are still supportive of the hypothesis that a disparity exists between these lines of chickens in the capacity of their cellular targets for neoplastic transformation in one or more parameters of direct interaction with MDV. An alternative explanation for the high losses seen after transfer of line 7 adult spleen cells to line 6 (Table IV) would be that cells suppressive of a protective immune response against MD were present in transferred line 7 cells. MDV-induced suppressor cells have been described by Lee and her co-workers (1978b). If present in the experiments reported here, however, suppressor cells would have to be relatively radio-resistant as a high frequency of MD was recorded in the line 6--> line 7 combination (group C). Significantly, no protective effect was observed upon reciprocal transfer of adult, SPF, line 6 spleen cells into irradiated line 7 recipients.

That the cellular characteristic required for

susceptibility may be associated with a particular stage of T-lymphocyte maturation is evidenced by the fact that adult and embryonic spleen are not equally efficacious in increasing susceptibility upon adoptive transfer. Alternatively, the relevant target cells may be present during embryogenesis but may not be localized in the embryonic spleen at the stage during which our adoptive transfers were performed.

#### C. The Effect of Thymic Microenvironment on HVT Adsorption

It is, of course, well established that T-cells migrate through and undergo a large portion of their maturation within the confines of the thymus (reviewed by J. Miller, 1980). It is unlikely that the lymphocytes present in the thymus grafts after 7 days on the CAM were intrinsically derived from radioresistant cells remaining in the grafted fragments. First, thymic lymphocytes and their precursors are extremely sensitive to the dose of irradiation we employed in these experiments (Trowell, 1962; Montour, 1967). Second, chromosome marker studies in chick embryos have shown that grafts of normal embryonic thymus are repopulated by circulating host lymphocyte progenitors (Moore and Owen, 1967a). Also following reconstitution of 800R irradiated chick embryos with 14 day embryonic spleen the dividing lymphocytes of intact host thymuses were found to be primarily of extrinsic donor origin (Moore and Owen, 1967b). In the mouse it has been shown that at lower doses

of irradiation (400R) thymic regeneration is biphasic with the first phase being intrinsically derived from radio-resistant cells remaining in the thymus. At higher doses of irradiation (500-550R) this first phase of regeneration was markedly decreased (Takada et al., 1969). Finally, at high doses of irradiation (2000R) no donor origin mitoses were observed from 2-22 days post-grafting in CBA thymuses grafted onto CBAT6/T6 host mice (Dukor et al., 1965). One can, therefore, reasonably assume that the vast majority of the lymphoid fraction of the 7 day old grafts reported here were the progeny of host stem cells. The availability of unambiguous serological markers for the origin of intrathymic lymphocytes would, of course, help determine the relative numbers of host and donor cells in the thymus. The possible contribution of rare donor line 7 cells with a high virus adsorption capacity to the overall virus binding phenotype of these grafts would be difficult, if not impossible, to satisfactorily exclude even with such markers.

With these considerations in mind, the data from Figure 4 show that thymocytes of line 6 host origin, which mature in the microenvironment of a line 7 thymic stromal graft, have an enhanced capacity to adsorb HVT compared to thymocytes residing in line 6 grafts. This enhancement could be the result of some change at the level of individual target cells with respect to virus receptor structure or number. Alternatively, the cells adsorbing virus might be

qualitatively identical regarding receptor function and density, but the number of cells in this category could be somewhat expanded during maturation in a line 7 thymic microenvironment. In order to determine if one or both of these mechanisms are operative, the frequency of virus-binding cells in the grafts must be assayed. In addition, the amount of virus bound per cell should be quantified with  $^{125}\text{I}$  and fluorescein-labelled monoclonal anti-HVT probes. It is important to note that while the line specific difference in virus adsorption by thymic lymphocytes may be conferred by their exposure to the thymic stromal cells, this difference is not a property also displayed by the stromal cells themselves (Figure 5).

It was of interest to determine whether a resistant line 6 thymic environment would exert a reciprocal inhibitory effect on line 7 stem cells. To accomplish this one would first have to graft line 6 and 7 thymic stroma onto the CAM's of line 7 eggs. Experiments of this type were underway during the Fall of 1980 when it was discovered by accident that the "B<sup>2</sup>/B<sup>2</sup>" line 6 breeding stocks contained B haplotypes other than B<sup>2</sup> (B.M. Longenecker, personal communication), making the proposed experiments impossible to complete.



#### D. Monoclonal Antibodies Against HVT

Difficulties were encountered in attempts to enumerate virus-binding cells in line 6 and line 7 using conventional chicken anti-HVT reagents. To surmount these problems monoclonal antibodies against HVT antigens were developed. Monoclonal antibodies against antigens associated with two other oncogenic viruses, the Epstein-Barr virus and Simian virus-40 have recently been described in the literature. (Hoffman et al., 1980; Gurney et al., 1980). To my knowledge the antibodies reported in this study HVT.1 and HVT.2, represent the first monoclonal antibodies against HVT, the vaccine virus for Marek's disease. As such they represent a significant advance in the technology available for characterization of HVT and possibly MDV-related antigens; definition of virus products in this system has been hindered heretofore by a lack of truly monospecific antisera.

In initial tests with HVT.1 and HVT.2 labelled directly with fluorescein, nonspecific, cytoplasmic staining of fixed CEF was a problem. There was a good possibility that HVT.1 and HVT.2 defined HVT antigens expressed on the cell surface so another approach was used. Advantage was taken of the fact that fluorescinated latex beads were available which were too large to penetrate the plasma membrane of fixed cells. Beads coated with either HVT.1 or HVT.2 bound only to morphologically distinct HVT-induced foci in infected CEF (Figures 9a-14b). As another control for nonspecific

binding, beads coated with 7H.3, an IgG antibody which is specific for human lymphocytes were also tested. No binding was observed (Figure 15a-b). Since both HVT.1 and HVT.2 also neutralized cell-free HVT and any given monoclonal antibody has but a single combining specificity, it can be concluded that HVT virions and the membranes of infected CEF do share antigenic determinants. Common antigens of this sort have been suggested indirectly by a number of other studies using hyperimmune sera (reviewed by Nazerian, 1980), but, due to the complexity of these reagents, no definite conclusion could be reached. Whether HVT.1 and HVT.2 detect the same antigen or not has yet to be determined.

It is also of interest that even at the highest concentrations of antibody tested a significant fraction (approximately 20%) of HVT was not neutralized (Figures 7 and 8). Similarly, a small percentage of HVT foci did not bind HVT.1 or HVT.2-coated latex beads (data not shown). One possible explanation for these observations would be that the HVT stock used was antigenically heterogeneous. If this is so, then the restricted specificity of monoclonal antibodies such as HVT.1 and HVT.2 may prove useful for selection of antigen loss mutants in this system.

### E. MDV Infection In Vivo in Irradiated Recipients of Line 6 and 7 Spleen Cells

Given the obvious fluctuations which occur with time in the number of in vivo infectious centres seen in peripheral blood of the third-party irradiation chimeras (Figure 19), the results of these experiments are difficult to interpret. It is clear, nonetheless, from both Figure 17 and Figure 19 that at certain time points the numbers of MDV-infected cells in both spleen and peripheral blood are much higher in the line 7 group than in the line 6 group. The reverse situation does not occur at any sampling time. One possible interpretation of Figure 19 would be that between 10 and 17 days post-reconstitution the line 6 recipients were more capable of eliminating infected cells. A longer time course study will be required to determine if the decrease in infectious centres in the line 6 group at this time point is reproducible. This result may only reflect cyclic variations in virus load which are not causally linked to immune surveillance mechanisms.

The data presented in table 5 indicate that for adult spleen cells, peripheral blood lymphocytes and thymocytes there is a consistently higher frequency of virus binding cells in the line 7 birds. Spleen and peripheral blood lymphocytes of both lines showed a higher frequency of virus binding cells than did thymocytes. The line specific differences in the frequencies observed for each organ were also greater in the former two cell types. This is the

expected result if a mature T-lymphocyte is the target for MDV. A comparison of the line specific differences in virus adsorption as measured by the two assays used in this project, FIA and ADRA, does, however, yield an interesting discrepancy. Upon casual inspection the magnitude of the differences in virus binding cell frequency as measured by ADRA appears to be greater than one would expect on the basis of the result from the focus inhibition assays. In some instances, notably in tests of thymus tissue, these discrepancies in the ratios of adsorption by line 6 and line 7 are only about 2-fold and except for the reproducibility of this difference could be explained by experimental error. In the case of tests with spleen cell preparations, however, the separation is much larger, 20-fold with ADRA vs 2-fold with FIA and not easily explained by trivial variations in the techniques involved. Actually, the data from these two assays are not inconsistent with each other if one considers more carefully how the data from the FIA tests have been graphically presented. In Figures 2 and 3 virus foci are plotted as a function of the cell number used for adsorption. A fixed amount of input virus was used for each sample. The most valid comparison would be to compare the cell numbers required to adsorb 50% of the input virus. When the FIA data are examined in this manner the differences between line 6 and line 7 are much larger and quite compatible with the results from the ADRA experiments of table 5. At least two additional possibilities exist to

account for these results. First, the ADRA assay may be more sensitive than the focus inhibition assay. If anything, one would expect the opposite to be true as ADRA is more likely than FIA to be dependent on the amount of virus bound to the target cell. Second, there is the possibility that two or more qualitatively distinct virus binding cell types exist within the spleen with different densities of virus receptor expression. If such were the case, the measurements of virus adsorption at the level of the entire spleen population could easily underestimate line specific differences present in the less frequent, but possibly more important, subpopulations. A less sensitive but more selective assay with a higher threshold for positives such as ADRA might then be expected to give greater resolution of these minor subpopulations.

#### F. Conclusions and Possible Interpretations

Two separate lines of research have been presented in this thesis. First it was demonstrated that line 6 and line 7 spleen, thymus and peripheral blood lymphocytes differ in their permissivity to infection by HVT in vitro. Second, it was discovered that susceptibility could be increased in line 6 birds by transfer of line 7 cells from organs known to contain T-lymphocytes. MD-specific mortality was also increased by transplantation of irradiated line 7 thymuses. These experiments are inconsistent with the behavior expected of conventional Ir genes controlling a favorable

immune response. A minimal target cell hypothesis predicts that the lymphocyte progeny of line 6 and 7 stem cells will inherently express differences in MDV replication/transformation irrespective of the genotype of the host in which they mature. In strictest terms, the data presented here are also at variance with this hypothesis. These results can, however, be accommodated provided one modifies the original model to allow for an inductive effect of the thymic microenvironment on the target cell pool.

The mechanism by which the thymic microenvironment exerts its effect on MD susceptibility has yet to be determined. The simplest explanation would be that in line 7 there is a greater overall production of T-lymphocytes and hence a greater number of potential target cells for MDV. Indeed, this idea is consistent with the performance of line 7 lymphocytes in a variety of T-dependent immunoassays (see Project Rationale). Alternatively, MDV may preferentially infect/transform a subset of the total T-cell pool (helper T-cells, for example). Overproduction of this subpopulation by line 7 in comparison to line 6 would also be expected to result in increased susceptibility. Selective expansion of T-cell clones with immunological receptors capable of binding MDV as antigen could also be considered as a possible explanation (Irving L. Weissman, personal communication). Such clonal amplification could be postulated to occur via an antigen driven mechanism if line 7 thymic stromal cells carry surface determinants

cross-reactive with MDV antigens. The frequency of virus binding cells recorded in Table V, 46 per 1000 for line 7 spleen, is, however, higher than what one would normally expect in an immunologically naive animal if such binding was mediated by an antigen-specific receptor.

In regard to the possibility of an inductive effect of the thymic microenvironment, in line 7 an analogy can be made with the Ir genes of the mouse. It has been suggested that populations of low responder stem cells can be converted to the high responder phenotype by an F1 thymic microenvironment (reviewed by J. Miller, 1980). Here again, the molecular mechanism of this effect is unknown. One does not know if there is selective expansion of a given T-cell clone(s) which exists in both high and low responder strains or if there is a qualitative change occurring in individual cells. Also, by analogy with the line 6 thymus, one does not know at this point whether a low responder's thymic microenvironment exerts a negative effect on T-cell maturation or simply fails to exert the required inductive influence.

Until more is known about the molecular aspects of the thymic environment's influence on MD susceptibility, the above argument will always be a problem. It is only in the murine leukemia virus (MULV) systems, where the molecular biology is well characterized, that such thymic influences are beginning to be understood. Datta, Waksal and Schwartz (1980) recently proposed that a phenotypic change in MULV

target cells occurs under thymic influence. They suggested that the thymic microenvironment fosters the generation of the xeno-ecotropic recombinant viruses thought to be the proximal agents in leukemogenesis. In the MD system the explanation is probably a different one.

Precedent for host genetic control of herpesvirus replication at the cellular level has recently been presented in the murine cytomegalovirus system (Nedrud et al., 1979). Also, the results recorded with thymus transplants have recently been confirmed by another group (P.C. Powell personal communication). The virus adsorption results have since been corroborated by Lee et al. (1980a). In agreement with the findings presented here, Powell et al. (1980a) have also argued that resistance to MD in lines 6 and 7 may be complex, having both an early component of target cell variability as well as immune surveillance mechanisms involved.

In this author's opinion, a clear understanding of non-MHC-associated genetic resistance to MD will require a more molecular approach. Monoclonal probes against HVT and lymphocyte surface markers could be used to select T-cell subpopulations for further biochemical characterization. They might also be useful for the isolation of the virus receptor itself, but this would probably be technically difficult. They should also be used in fluorescinated form to determine the number of virus binding cells in CAM thymus grafts.



In order to prove the link between HVT adsorption phenotype and capacity to increase MD susceptibility, additional experiments are necessary. One would have to physically select line 6 and 7 spleen cells on the basis of HVT adsorption and then use these cells for adoptive transfer in mortality studies. The HVT-1 and HVT-2 monoclonal reagents could be used in fluorescinated form in conjunction with a fluorescence activated cell sorter to accomplish this selection. Of course, one might have to partition the virus binding cells into subsets according to the amount of HVT bound. More than one virus-binding cell type may exist. One does have to remember as well that MDV may not spread in vitro by such an adsorption mechanism but rather by cell to cell contact. Adsorption phenotype may, thus, serve only as a useful marker.

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