HOKKAIDO UNIVERSITY

Instructions for use

ANTI-TUMOR IMMUNITY AGAINST MAREK'S DISEASE-DERIVED LYMPHOBLASTOID CELL LINE (MSB-1)*

Chihiro Sugimoto, Hiroshi Kodama and Takeshi Mikami

Department of Epizootiology
Faculty of Veterinary Medicine
Hokkaido University, Sapporo 060, Japan

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Cytotoxic effects of peripheral blood lymphocytes (PBL) and immune sera from chickens infected with Marek's disease virus (MDV) or vaccinated with herpesvirus of turkey (HVT) against Marek's disease lymphoblastoid cell line (MSB-1) cells were studied. Cellular immunity was assayed using lymphocytes collected 60 to 180 days postinfection. Lymphocyte cytotoxicity tests revealed that the PBL of MDV infected chickens had cytotoxic effects against MSB-1 but those of HVT-vaccinated chickens did not. In complement-dependent antibody cytotoxicity (CDAC) and antibody-dependent cell-mediated cytotoxicity (ADCC) tests, no cytotoxic activity of sera from MDV- or HVT-infected chickens was demonstrated. However, after the sera of chickens immunized with inactivated MSB-1 cells were extensively absorbed with chicken red blood cells, these sera had cytotoxic effects against MSB-1 cells in CDAC and ADCC tests.

INTRODUCTION

Marek's disease virus (MDV) causes lymphomas in chickens, but vaccination with herpesvirus of turkey (HVT) suppresses the occurrence of Marek's disease (MD) lymphomas. Lymphoblastoid cell lines have been established from MD lymphomas,[2,14] and these cell lines are thought to be of T cell origin.[5,14] A tumor specific antigen designated the MD associated tumor surface antigen (MATSA),[20] has been noted on the surface of the cells, and MATSA has also been detected in lymphomas in vivo.[6] Immunization with glutaraldehyde-fixed cells bearing MATSA was protective against MDV challenge.[12] In recent studies, cell-mediated cytotoxicity against MD lymphoma cell lines using spleen cells or buffy coat cells from chickens infected with MDV or immunized with glutaraldehyde-fixed MD lymphoblastoid line cells has been demonstrated and has been thought to be directed against MATSA.[1,13,15,18,19] It is important to consider the possibility of immunological surveillance against MATSA in anti-tumor immunity in MD and in the mechanism of vaccinal (HVT) immunity. In this study cell-mediated and humoral immunity against the MD lymphoblastoid cell line (MSB-1) cells were examined. The

purpose of these experiments was to evaluate the roles of both types of immunity in their resistance to MD lymphoma formation.

MATERIALS AND METHODS

Animals and viruses

Chicks were obtained from a specific pathogen free (SPF) flock of White Leghorns (Line M, Nippon Institute for Biological Science, Tokyo, Japan). The chicks were free of maternal antibodies of MDV or HVT.

The JM strain of MDV and the FC 126 strain of HVT were used; the sources and the methods of propagation of these viruses were described by MIKAMI & BANKOWSKI (1970) and OKADA et al. (1972).

A group of day-old chicks was inoculated intraperitoneally with 0.1 ml of heparinized blood (titer; 30~40 plaque forming units (PFU)/0.1 ml) from chickens infected with MDV. A second group of day-old chicks was inoculated intraperitoneally with 230 PFU (0.1 ml) of HVT infected chick kidney cells (CKC). A third group of non-inoculated chicks served as controls. The groups were kept in separate rooms. Maximum precautions were taken to prevent cross contamination among the three groups of chickens. The infection status was monitored in each group by direct kidney cultures, virus isolation from peripheral blood lymphocytes (PBL) and antibody detection using the agar gel precipitin (AGP) test.

Cell culture

The MSB-1 cell line, which was established from splenic lymphoma,[2] was kindly provided by Dr. S. KATO (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). The MSB-1 cells were cultured in RPMI-1640 with 10% fetal calf serum (FCS), penicillin (200 U/ml), streptomycin (200 μg/ml), and fungizone (2.5 μg/ml), at 41°C in an atmosphere containing 5% CO_2, and subcultured every 2 days.

The 1104-X-5 cell line was derived from a bursal lymphoma of a chicken inoculated with subgroup A avian oncornavirus.[3] This cell line was given by Dr. H. HIHARA (National Institute of Animal Health, Tokyo, Japan). The cells were originally cultured in Eagle's minimum essential medium (MEM) with non-essential amino acids at 37°C, but we adapted them to the culture condition used for the MSB-1 cells. RPMI-1640 containing 10% FCS and antibiotics was used throughout the experiments.

Sera

Sera were collected from MDV-infected, HVT-vaccinated and non-infected chickens of various ages and stored at −20°C. Antibodies to MDV or HVT were detected by the AGP test.[17] Antigens were prepared from the skins of chickens infected with MDV or from supernatant fluids of HVT-infected CKC cultures.[9] The titers of the sera from infected chickens against the homologous antigens varied from ×1 to ×8.

The sera from the non-infected chickens were negative.

Hyperimmune serum against the MSB-1 cells was prepared in the chickens by 4 inoculations of glutaraldehyde-fixed MSB-1 cells $(2.5 \times 10^6 - 4.3 \times 10^7$ cells). The first inoculation was made with complete Freund's adjuvant (Iatron Laboratories, Tokyo, Japan), and the serum was collected 10 days after the last inoculation. All sera were inactivated at 56°C for 30 minutes before use.

Before using the complement-dependent antibody cytotoxicity (CDAC) and the antibody-dependent cell-mediated cytotoxicity (ADCC) tests, anti-MSB-1 serum was repeatedly absorbed with an equal volume of packed chicken red blood cells at room temperature for 1 hour to remove reactivity against normal cellular antigens. The chicken red blood cells were obtained from a pooled peripheral blood sample from more than 20 chickens.

^{51}Cr labelling method of target cells

Two-day-old cells (MSB-1) or 3-day-old cells (1104-X-5) were harvested, pelleted and resuspended in 1.5 ml of fresh culture medium at concentrations of 2 to 4×10^7 cells/ml. One hundred μCi of $Na_2^{51}CrO_4$ solution (specific activity ; 100 to 400 mCi/mg of chromium, concentration ; 1 mCi/ml, The Radiochemical Centre, Amersham, England) was added to the cell suspension, and the mixture was incubated for 2 hours for the MSB-1 cells and 1 hour for the 1104-X-5 cells at 41°C for labelling with ^{51}Cr.

After incubation the cells were washed 4 times with Dulbecco's phosphate-buffered saline (PBS), put into ice for 30 minutes, rewashed, and finally resuspended in the culture medium.

Lymphocyte cytotoxicity test

In each experiment heparinized blood was collected from chickens of the same age in the three different groups. The lymphocytes were separated from the Ficoll-Conray mixture by centrifugation at 500 g for 30 minutes, then washed once with PBS and resuspended in the culture medium at concentrations of 2 to 20×10^7 cells/ml. The Ficoll-Conray mixture consisted of 2.4 parts of 9% Ficoll 400 (Pharmachia Fine Chemicals, Uppsala, Sweden) solution in distilled water and 1 part of 33.4% Conray (Daiichi Seiyaku Co., Tokyo, Japan). The mixture of 1 ml of the ^{51}Cr labelled target cell suspension $(2 \times 10^5$ cells/ml) and 1 ml of one of the lymphocyte suspensions was incubated at 41°C for 10~18 hours in a water bath and shaked continuously. After incubation, samples were centrifuged at 600 g for 5 minutes, and 1 ml of the supernatant was collected and counted for radioactivity in a well-type scintillation counter (PS-9, Japan Radio Co., Tokyo, Japan).

Complement-dependent antibody cytotoxicity (CDAC) test

The mixture of 0.05 ml of ^{51}Cr labelled cell suspension $(4 \times 10^6$ cells/ml), 0.05 ml of antiserum, and 0.05 ml of duck or chicken complement was incubated for 1 hour at

37°C. After incubation the mixture was placed on ice, and then 0.85 ml of cold PBS (0.05 M, pH 7.0) containing 10 mM EDTA was immediately added to the mixture. Following centrifugation at 600 g for 5 minutes, 0.5 ml of the supernatant was collected and counted for radioactivity.

Fresh duck serum, which had been absorbed with 10^8 MSB-1 cells per 1 ml of serum at 0°C for 30 minutes and diluted ×2 with the culture medium, was used as the duck complement. Fresh chicken serum obtained from SPF chickens was used as the chicken complement. Both sera were free of antibodies against MDV or HVT as determined by the AGP test.

Antibody-dependent cell-mediated cytotoxicity (ADCC) test

Heparinized peripheral blood was obtained from 120 to 210 day-old normal chickens, and the lymphocytes were separated by the Ficoll-Conray mixture as described. The mixture of 1 ml of ^{51}Cr labelled target cell suspension (2×10^5 cells/ml), 0.5 ml of test serum, and 0.5 ml of normal lymphocyte suspension was incubated for 12 hours at 41°C. After incubation, the samples were centrifuged at 600 g for 5 minutes, and 1 ml of the supernatant was collected and counted for radioactivity.

Calculation of specific release and percent release of ^{51}Cr and statistical analysis

The specific release of ^{51}Cr was calculated using the following formula: $\frac{E-\bar{N}}{T-\bar{N}} \times$ 100 (%): where $E=$ counts per minute (cpm) of released ^{51}Cr in the test sample; $\bar{N}=$ average cpm of released ^{51}Cr in the control samples incubated with normal PBL in the lymphocyte cytotoxicity tests, with normal serum and complement in the CDAC test, or with normal serum and normal PBL in the ADCC tests; and $T=$ cpm of total incorporated ^{51}Cr.

The percent release was calculated as $E/T \times 100$ (%). Analysis of the results was made by variance and the Student's t-test.

RESULTS

Lymphocyte cytotoxicity test

The lymphocyte cytotoxicity tests against MSB-1 cells with PBL from chickens inoculated with MDV, chickens vaccinated with HVT, and normal chickens were done 8 times. The age of the chickens from which PBL were collected varied from 60 to 180 days old. High cytotoxic activity against MSB-1 with PBL from the MDV infected chickens was detected in 4 experiments. Figure 1 (a, b) shows the results of two of these experiments. The cytotoxic activity was observed most clearly at an effector-target cell ratio of 100 : 1 after 18 hours incubation. Cytotoxic activity against MSB-1 with PBL from the HVT-vaccinated chickens was not clearly observed in the same experiments.

As control experiments, lymphocyte cytotoxicity tests against 1104-X-5 cells were done 6 times using PBL from 90 to 180-day-old chickens. No cytotoxic activity with PBL from the MDV-infected and HVT-vaccinated chickens was observed in any of the experiments. Figure 1 (c, d) shows the results of 2 experiments.

FIGURE 1   *Lymphocyte cytotoxicity against MSB-1 and 1104-X-5*
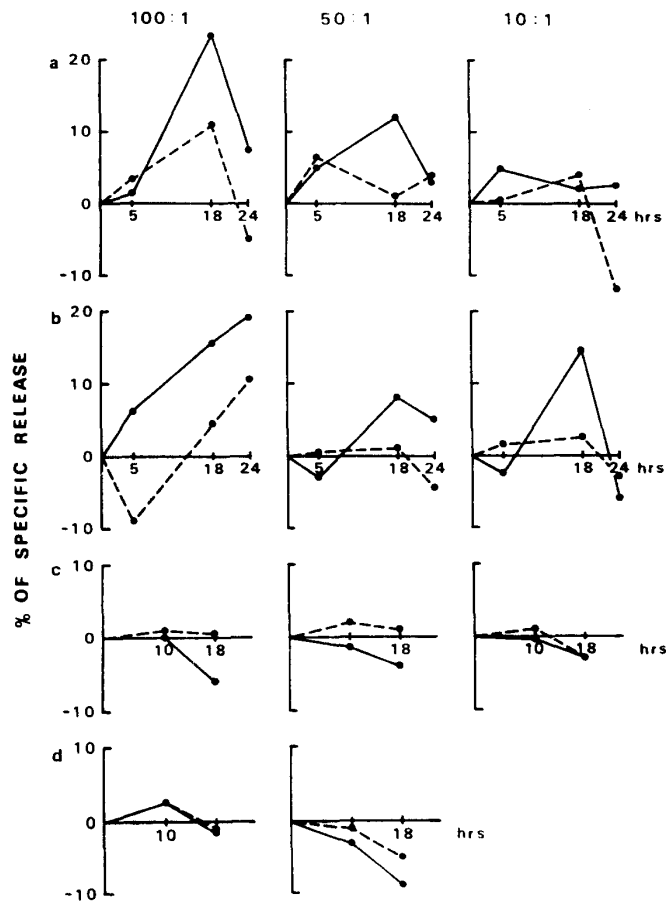
Lymphocyte cytotoxicity of PBL from MDV-infected (●—●) or HVT-vaccinated (●- -●) chickens against MSB-1 (a, b) or 1104-X-5 (c, d) at effector-target cell ratios of 100 : 1, 50 : 1 and 10 : 1. The age of chickens from which PBL were collected was 125 days old in a, 150 days in b, and 180 days in c and d.

The results obtained from all of the experiments are presented in table 1. The PBL of the MDV-infected chickens had a higher cytotoxic activity against MSB-1 than that of the HVT-vaccinated or the non-infected normal chickens at effector-target cell ratios of 100 : 1 and 50 : 1, but the difference was statistically significant only at 100 : 1 (P < 0.05). The cytotoxicity of PBL from the HVT-vaccinated chickens against MSB-1 was not significantly different from that of normal PBL.

There was no statistically significant difference between pairs of the three groups at any effector-target cell ratio against 1104-X-5.

CDAC test

The CDAC test was done by using either the duck or chicken complement. High

TABLE 1   *Mean specific release in lymphocyte cytotoxicity test*
against MSB-1 and 1104-X-5

| SOURCE OF EFFECTOR CELLS (PBL)	SPECIFIC RELEASE (%) MSB-1[a]			MEAN ± SE 1104-X-5[a]		
	100 : 1[b]	50 : 1	10 : 1	100 : 1	50 : 1	10 : 1
MDV-infected chickens	4.35 ± 4.46[c]	3.87 ± 4.70	2.70 ± 3.44	0.36 ± 1.76	−1.34 ± 1.88	−0.23 ± 2.39
HVT-vaccinated chickens	−0.25 ± 2.08	−0.32 ± 4.44	2.79 ± 5.04	1.16 ± 1.52	0.18 ± 2.12	1.10 ± 2.90
Normal chickens[d]	0.00 ± 2.50	0.00 ± 1.54	0.00 ± 2.46	0.00 ± 1.30	00.0 ± 1.66	0.00 ± 2.60

a   Target cells
b   Effector-target cell (E/T) ratio
c   Values are significantly different from those of the HVT vaccinated and normal chickens ($P<0.05$).
d   Values of % release of samples incubated with PBL from non-infected chickens were : for MSB-1 at 100 : 1=54.0 %, 50 : 1=54.0 % ; and 10 : 1=54.4 % ; and for 1104-X-5 at 100 : 1=21.8 %, 50 : 1=23.0 % and 10 : 1=24.0 %.

FIGURE 2   *CDAC of anti-MSB-1 chicken serum with duck*
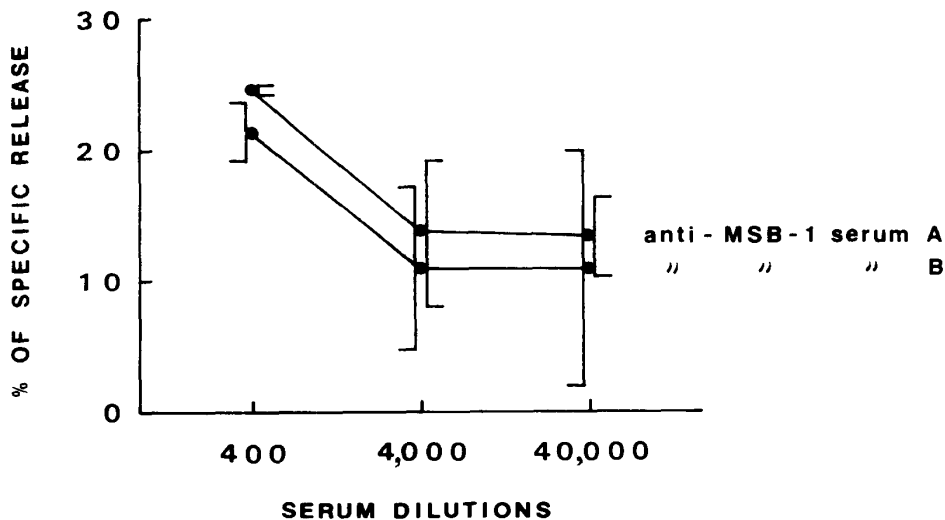*complement against MSB-1*
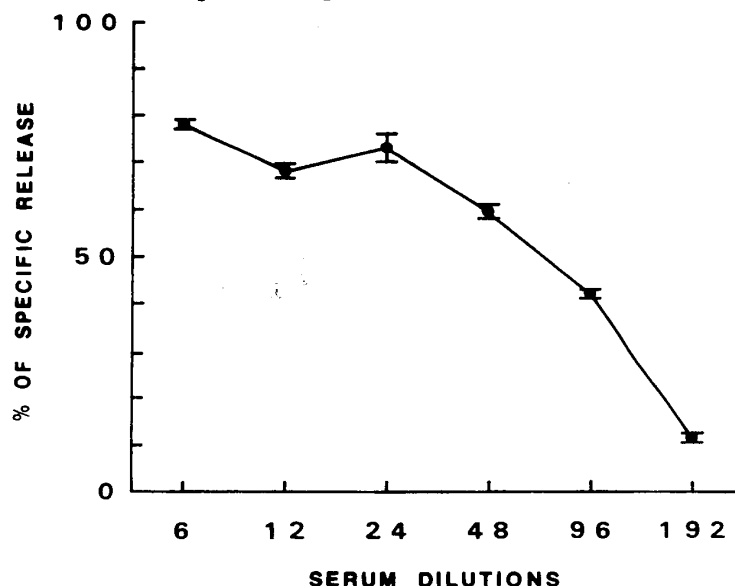
Result is shown as mean ± SD. Values of % release in control were : complement without serum=8.4 % ; normal serum with complement=9.9∼14.8 % ; and medium only=7.6 %.

FIGURE 3　CDAC of sera of MDV-infected (M), HVT-vaccinated (H), and normal chickens (N) with duck complement against MSB-1
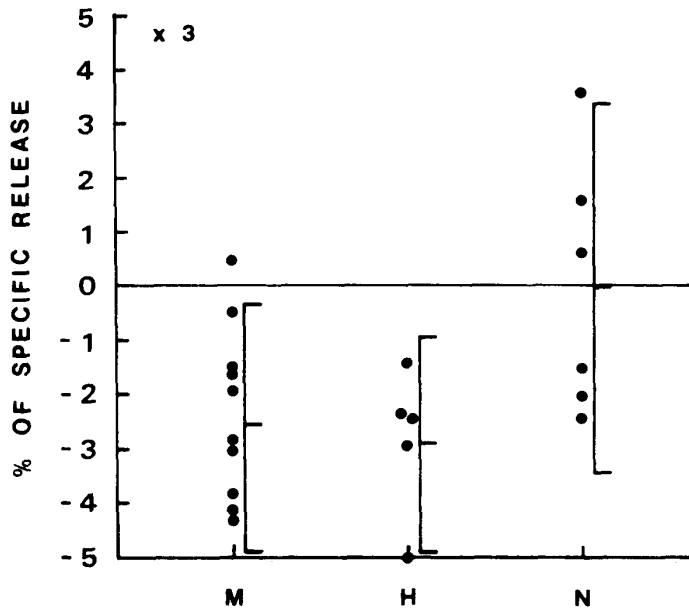
The final dilution of the sera tested was ×3. Points (●) show the means of duplicate samples of each serum, and to the right, the mean ± SE of each serum tested is shown. Values of the % release in the controls were : normal sera with complement = 9.0 % ; complement without serum = 14.5 % ; and medium only = 7.6 %.

FIGURE 4　ADCC of anti-MSB-1 chicken sera with normal PBL against MSB-1

Result is shown as mean ± SD. Values of % release in the control were : normal serum with PBL = 59.6～61.1 % ; PBL without serum = 64.0 % ; and medium only = 56.2 %.

cytotoxic activity of anti-MSB-1 chicken serum was demonstrated in the presence of the duck complement (fig. 2); however, complemental activity of the chicken complement was poor. Although more than 70% of specific release was observed at 1 : 6 dilution of the hyperimmune serum with the duck complement, only a few % of specific release was demonstrated at the same dilution of the serum with the chicken complement.

When sera from the MDV-infected, HVT-vaccinated, and normal chickens were tested for CDAC against MSB-1, no cytotoxic activity was observed, even though some of these sera had a titer of 1 : 8 against homologous antigens in the AGP test (fig. 3).

ADCC test

The ADCC test against MSB-1 was performed by using PBL from normal chickens aged 120 to 210 days. At an effector-target cell ratio of 50 : 1, the specific cytotoxic effect against MSB-1 was observed in the presence of anti-MSB-1 serum, even at a high dilution of serum (×40,000) (fig. 4). No ADCC, however, was observed at the ratio of 10 : 1 (this data is not shown).

The result of the ADCC tests with sera from MDV-infected, HVT-vaccinated chick-

FIGURE 5   *ADCC of sera of MDV-infected (M), HVT-vaccinated (H) and normal chickens (N) with normal PBL against MSB-1*
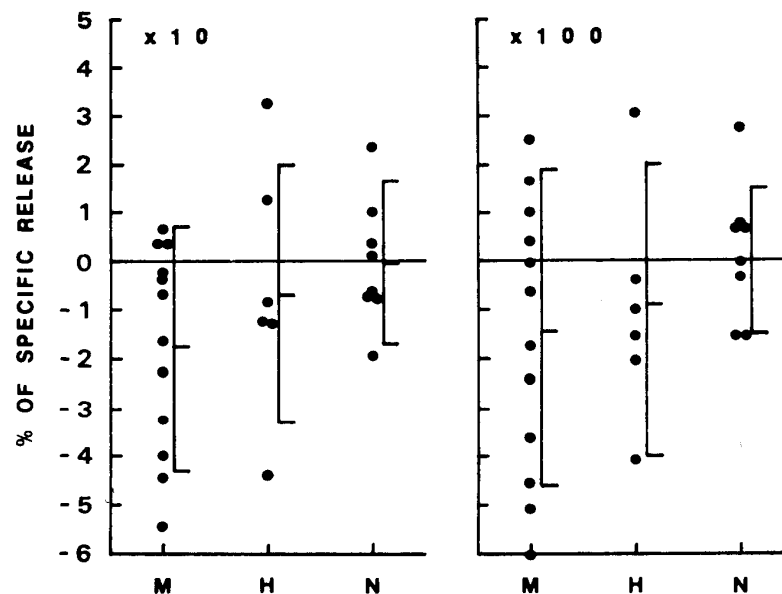
Refer to the appendix of figure 2. The test was done at two different dilutions (×10 and ×100) for each serum tested and at an effector-target cell ratio of 50 : 1. Values of % release in the control were: normal sera (×10) with PBL=47.5% ; normal sera (×100) with PBL=45.9% ; PBL without serum=51.2% ; and medium only=52.4%.

ens, and normal chickens is shown in figure 5. No ADCC was observed in any sera tested at dilutions of ×10 or ×100.

DISCUSSION

PAYNE et al. (1976) have proposed a "two step mechanism" in MD immunity, that is, a first step of host immune responses directed against MD viral antigens, and a second step directed against tumor antigens. As suggested by POWELL & ROWELL (1977), anti-viral and anti-tumor immunity appear to be dissociated from one another, and both types of immunity may be separately concerned with host resistance. In natural infection, both types may be closely related. Anti-viral immunity reduces the amount of virus,[15] liberates the host from immunosuppression by MDV, and preserves second step immunity against transformed cells.

In the present experiments cell-mediated and humoral immunity against MSB-1 cells were examined to evaluate the roles of both types of immunity in their resistance to MD lymphomas. Cell-mediated immunity was assayed with lymphocytes collected from 60- to 180-day-old chickens inoculated with MDV or HVT at one day of age. In line with all earlier publications,[1,13,19] the levels of cytotoxicity exhibited by lymphocytes from the MDV-infected chickens were very low, but apparently statistically significant. Lymphocytes from the HVT-infected chickens were inactive. The activity of the MDV-infected lymphocytes was detected over a prolonged age range. All these observations confirm the report of POWELL (1976), but conflict with that of SHARMA & COULSON (1977), who found only transient activity in the MDV-infected chickens 7~9 days after infection. SHARMA (1977) has recently reported similar cytotoxicity of spleen cells from HVT-infected chickens at 7 and 8 days post infection; however, ADLDINGER & CONFER (1977) reported that cytotoxic activity against MSB-1 cells was observed with lymphoid cells from chickens inoculated with normal, non-infected chicken kidney cells at 5 to 10 days after inoculation. A second peak in activity was observed at 2~3 weeks post inoculation, and this peak occurred only in chickens infected with MDV by contact. The early peak appeared to represent an allotypic response to the injected cells. Further studies, however, are necessary to clarify the early event of a transient cellular response in birds infected with MDV or HVT.

Although lymphocytes from MDV-infected chickens seem to be specifically cytotoxic to cells expressing MATSA, the possibility exists that this immune response may also be directed against other antigen(s) including membrane antigens (MA), histocompatibility antigens, and possibly others expressed on the surface of the MSB-1 cells. It is known, however, that the proportion of MSB-1 cells which express MA is very low (1 to 2%).[6,14] The Line M chickens used in the present experiments were not inbred; therefore, these chickens probably had different histocompatibility antigens at the B locus from those of the MSB-1 cells. However, cytotoxicity of normal PBL against the MSB-1

cells was lower than that of the MDV sensitized PBL, and the degrees of cytotoxicities of lymphocytes from MDV-infected and normal chickens against 1104-X-5 cells, which were unrelated to MD, were similar. Therefore, these possibilities can be ruled out. At the time of PBL collection, most of these chickens were healthy and showed no MD symptoms, but some of them were depressed or showed slight nervous symptoms. Although the cytotoxic activity of these PBL against the MSB-1 cells varied from one experiment to another, the degree of activity had no apparent relation to the severity of MD in chickens at the time of PBL collection.

The results obtained by the CDAC and ADCC tests, which indicated the absence of anti-MATSA antibodies in the MDV-infected birds, were in agreement with other results obtained by the membrane immunofluorescence assay test.[6,20] According to reports on other systems[11,16] the sensitivity of the ADCC test was higher than that of the membrane immunofluorescence. Although we cannot neglect the possibility that the antibody level was too low to be detected by these methods, it appears that MATSA may escape from the humoral immunity of an MDV-infected host because of its low antigencity to the chicken or a disturbance of T-cell-dependent antibody production, as discussed by MATSUDA et al.[6] Thus, it seems that humoral antibody does not play a major part in MD tumor immunity in MDV-infected chickens. Humoral cytotoxic activity was detected in the sera of chickens immunized with inactivated MSB-1 cells. Since the sera were extensively absorbed with chicken red blood cells before use, this activity was presumably directed against a "tumor antigen". However, it is possible that this activity may be directed against histocompatibility antigens on the MSB-1 cells. Further studies are required before the significance of this finding in tumor immunity can be determined.

Since MATSA was expressed on MD tumor cells in vivo,[6,20] cell-mediated immunity against MATSA may play an important role in MD immunity in vivo. The long lasting and low level cytotoxic response of the PBL of MDV-infected chickens may be responsible for the immune surveillance in MD. In the HVT vaccinated chickens, neither a cell-mediated nor a humoral immune response against the MSB-1 cells was found. Antiviral immunity of the HVT vaccinated chickens against cells productively infected with MDV was demonstrated by the ADCC test.[4] This result suggested that anti-viral immunity lowers the level of virus by protecting the host immune systems from the damage caused by MDV.

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