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CELL-MEDIATED CYTOTOXICITY OF LYMPHOCYTES FROM CHICKENS INOCULATED WITH HERPESVIRUS OF TURKEY AGAINST A MAREK'S DISEASE LYMPHOMA CELL LINE (MSB-1)

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SUMMARY Using a new device which increases the sensitivity of detection of specific immune lysis of target cells by labeling them with [^{35}S]-methionine, the in vitro cell-mediated cytotoxic response of spleen lymphocytes and peripheral blood lymphocytes from chickens vaccinated with herpesvirus of turkey (HVT), O1 strain, against MSB-1 line cells was clearly demonstrated. The cytotoxic activity was clearly inhibited by pretreatment of effector lymphocytes with anti-T lymphocyte serum and complement. The activity was greater using T cells purified from spleen lymphocytes and peripheral blood lymphocytes than with the unfractionated cells, indicating that T lymphocytes play the main role in effector activity. Using sera from HVT-vaccinated chickens, no significant cytotoxic effects were detected in the complement-dependent antibody cytotoxicity test against MSB-1 cells. These results suggest that cellular immunity against the surface antigen of Marek's disease (MD) lymphoma cells is mainly related to the preventive mechanism against MD incidence by HVT vaccination.

INTRODUCTION

Marek's disease (MD) is a naturally occurring malignant neoplasm of chickens caused by a herpesvirus named MD virus (MDV) and it is the only known malignant tumor that can be prevented by vaccination. For vaccination, herpesvirus of turkey (HVT) which is antigenically related to MDV is now extensively used as live vaccine virus. However, the mechanism of its protective effect is unknown.

Several workers, using the [^{51}Cr]-release technique, have demonstrated an in vitro cell-mediated cytotoxicity of spleen lymphocytes (SPL) (Sharma and Coulson, 1977; Sharma, 1977a, b) or peripheral blood lymphocytes (PBL) (Powell, 1976; Powell and Rowell, 1977; Adldinger and Confer, 1977; Sugimoto

et al., 1978) from chickens infected with various strains of MDV against MD lymphoma cell lines, such as the MSB-1 line (Akiyama and Kato, 1974) and the HPRS-1 and HPRS-2 lines (Powell et al., 1974), although the levels of cytotoxic activity in these experiments were not high. Furthermore, using the [^{3}H]-proline labeling technique, Dambrine et al. (1977) demonstrated considerable cytotoxic activity of SPL obtained from MDV-infected chickens against MSB-1 cells. All these experiments show that MDV-infected chickens develop a cell-mediated immune response to MDV-transformed cells expressing the MD tumor-associated surface antigen (MATSA) demonstrated by Witter et al. (1975).

On the other hand, there are several reports of studies using the [^{51}Cr]-release technique on the in vitro cell-mediated cytotoxicity of lymphocytes from HVT-inoculated chickens to MDV-transformed cells (Powell, 1976; Sharma, 1977b; Sugimoto et al., 1978). Sharma demonstrated low but significant cytotoxic activity of lymphocytes of HVT-inoculated chickens against MSB-1 cells, but the other authors failed to detect this activity. The discrepancy between these results may be due to differences in experimental conditions.

This paper reports studies on the in vitro cell-mediated cytotoxic response of lymphocytes from HVT-inoculated chickens to MSB-1 cells examined by the [^{35}S]-methionine labeling technique.

MATERIALS AND METHODS

1. *Chickens*

The chickens used were from a specific pathogen free (SPF) flock of SPAFAS (COFAL negative, SPAFAS, Inc., R.F.D. No. 3, Norwich, Conneticut U.S.A. Eggs were kindly provided by Bio Pharmaceuticals Inc., Tokyo). They were confirmed to be free from antibodies to HVT and MDV, and were kept under isolated conditions from the day of hatching throughout the experiments.

2. *HVT vaccination*

The O1 strain of HVT (Ono et al., 1974) was used. The virus was propagated in chick embryo fibroblasts (CEF) and infected cells were stored at −70 C until use. Two-week-old chickens were inoculated intraabdominally with 5,000 plaque-forming units (PFU) of HVT/0.2 ml/chicken. Uninoculated broodmates and, in some cases, CEF-inoculated broodmates were used as controls.

3. *Effector cells*

In each test 3 chickens from the HVT-inoculated group and an equal number of uninoculated broodmates and, in some cases, CEF-inoculated broodmates were examined simultaneously. For preparation of effector cells, these chickens were bled by cardiac puncture and the spleens and heparinized blood were collected aseptically. Decapsulated spleen tissues were chopped into small pieces with scissors and suspended in growth medium (RPMI-1640 supplemented with 10% fetal calf serum). The cell suspension was passed through a stainless-steel wire sieve. SPL and PBL were clarified by layering them on a Lymphoprep (Nyegaard & Co, Oslo, Norway) and centrifuged at 1,500 rpm for 20 min, and then they were washed three times with growth medium. Lymphocytes consisting of 80–93% viable cells, determined by the trypan blue exclusion test, were resuspended at an appropriate concentration.

4. *Target cells*

An MD lymphoma cell line named MSB-1 (Akiyama and Kato, 1974) was used as target cells. The conditions for maintenance of the MSB-1 cells have been described in detail (Akiyama and Kato, 1974). After 24 hr of subculture in growth medium (RPMI-1640+10% fetal calf serum), MSB-1 cells were washed three times with PBS, and resuspended at a concentration of 1×10^{7} cells/ml in 1 ml of growth medium without methionine and were labeled with 20 μCi of L-[^{35}S]-methionine (specific activity; 1,030 Ci/mmole, concentration; 1 mCi/20 ml; Radiochemical Centre, Amersham, England). After 3 hr incubation at 41 C, cells were washed three times with growth medium and resuspended at an appropriate concentration.

The 1104-B cell line (Hihara et al., 1974) was used as a control. This was derived from a lymphoma of the bursa of Fabricius of a chicken inoculated with subgroup A avian leukosis virus and

kindly provided by Dr. Hihara of the National Institute of Animal Health, Tokyo. The cell line has been shown to be B cell-derived and free from MATSA (Matsuda et al., 1976a).

5. *Cell-mediated cytotoxicity (CMC) test*

The optimum conditions for the CMC test were determined as described in the results. The standard procedure finally adopted for the test was as follows: 0.1 ml of target cell suspension (5×10^4 cells) was mixed with 0.1 ml of effector cell suspension (2.5×10^6 cells) of SPL or PBL at an effector-target (E/T) cell ratio of 50: 1. The test was carried out in a tightly stoppered test tube at 41 C in a water bath with gentle shaking for 36 hr. At least 3 tests were made on each sample. After incubation, the entire contents of each tube (0.2 ml) were applied to a quantitative filter (Whatman No. 542, Springfield Mill, Maidstone, Kent, England) and filtered by suction. The test tube and filter were washed with an additional 2 ml of PBS, and then the filter was washed 5 times with 2 ml of PBS. The surviving target cells on the filter were treated with 5% TCA and ethanol-ether solution (1: 1, by volume). The filters were transferred to scintillation vials, liquid scintillator (5 g PPO/1 toluene) was added and radioactivity was counted in a beta-scintillation counter (LS-150, Beckman Instruments Inc., Irvine, California). Average counts per minute (CPM) were determined and the specific release of [^{35}S] was calculated by the following formula:

$$\text{Specific cytotoxicity } (\%) = \frac{A-B}{A}\times100$$

where A = residual CPM with lymphocytes from untreated chickens or from chickens inoculated with uninfected CEF. B = residual CPM with lymphocytes from HVT-vaccinated chickens.

6. *Pretreatment of effector cells with antisera*

Specific antisera against normal thymus cells (anti-T serum) and normal bursa cells (anti-B serum) were prepared in rabbits, as described by Matsuda et al. (1976a). Immune sera were inactivated by heating, absorbed with chicken erythrocytes and repeatedly cross-absorbed with thymus or bursa cells from 2-week-old chickens until cross-reactivity was reduced to the background level. Samples of preimmunization serum (normal serum) were collected before immunization.

The specificities of anti-T and anti-B sera were examined by the indirect immunofluorescent (IF) test and complement-dependent antibody cytotoxicity test described by Matsuda et al. (1976a).

Effector cells were pretreated as described by Sharma (1977a). Briefly, SPL and PBL were treated with a 1: 10 final dilution of antiserum for 30 min at 37 C. The cells were then washed once, resuspended in 50 μl of growth medium, and incubated at 37 C for an additional 30 min with an equal volume of a 1: 10 final dilution of guinea pig complement. The cells were then washed 5 times and used in the CMC test at an E/T ratio of 50: 1.

7. *Purification of T cells by affinity chromatography*

SPL and PBL were fractionated as described by Schlossman and Hudson (1973). Briefly, 1 g of Sephadex G-200 (Pharmacia, Uppsala, Sweden) was washed with saline, brought to pH 10.5 with 0.2 N NaOH, and treated with 200 mg of cyanogen bromide (final concentration 2 mg/ml) for 10 min, maintaining the pH at 10.5 by dropwise addition of 1 N NaOH. The activated Sephadex was washed with 1,000 ml of borate-buffered saline, pH 8.3, mixed with 20 mg of purified rabbit anti-B serum with occasional stirring for 4 hr and finally washed with PBS. Glass wool was placed in the top of a disposable syringe (12 ml) and the tube was packed with 8 ml of Sephadex conjugate. The resulting column was washed with 100 ml of sterilized PBS and 30 ml of Eagle's minimum essential medium (EMEM) containing 5% fetal calf serum and 2.5 mM EDTA, incubated for 30 min at 37 C and returned to 4 C for cell fractionation. Suspensions of SPL and PBL cells that had been incubated for 3 hr at 37 C were adjusted to 6×10^7 cells/ml with EMEM, passed down the column and washed through with 20 ml of EMEM. The effluent cells were collected and used for the CMC test. The specificity of the purified T cells were examined by the indirect IF test with anti-T and anti-B sera.

8. *Complement-dependent antibody cytotoxicity (CDAC) test*

Cytotoxicity tests with chicken anti-MSB-1 and anti-HVT sera were carried out as follows. Sera were diluted with gelatin veronal buffer with Ca^{++} and Mg^{++} consisted of isotonic veronal buffer containing 2% gelatin, 2.5×10^{-4} M Ca^{++} and 8.3×10^{-4} M Mg^{++}, pH 7.4 (GVB^{++}) and 25 μl volumes of serial two-fold dilutions of each antiserum were incubated with 25 μl of [^{35}S]-methionine

labeled MSB-1 cell suspension (2×10^6 cells/ml/GVB^{++}) and 25 μl of duck complement (1:4 dilution in GVB^{++}) in the Microtest plate (3040 Microtest II, Falcon, Oxnard, California) for 60 min at 37 C. Then the mixtures were collected and treated as described above and radioactivity was measured. The cytotoxic index (CI) was calculated by the following formula:

$$\text{CI (\%)} = \frac{\text{mean residual CPM in controls} - \text{mean residual CPM in test samples}}{\text{mean residual CPM in controls}}$$

Sera used for the CDAC test were collected from HVT-vaccinated chickens. Three chickens were vaccinated with 5,000 PFU of HVT at 2 weeks of age. Four weeks later, their sera were pooled. The titer of antibody to HVT in this serum was >512, estimated by the indirect IF test with HVT-infected CEF.

Immune serum against MSB-1 cells, prepared in chikens as described by Matsuda et al. (1976b), was used as a positive control. Before use for the CDAC test, anti-MSB-1 serum was extensively absorbed with a mixture of bursal, thymic and splenic cells from normal chickens. The titer of the antibody to MATSA was×16 when titrated by the indirect IF test with MSB-1 line cells.

RESULTS

1. *Optimum conditions for the CMC test using [^{35}S]-methionine labeled target cells*

Five factors were found to influence the specific release of [^{35}S], as described below. To determine the optimal conditions for specific release of [^{35}S], the effects of these factors were each examined. Chickens were inoculated with HVT at 2 weeks of age, and 2–6 weeks after inoculation, SPL from 3 inoculated chickens were examined by the CMC test against MSB-1 cells by the standard procedure (see Materials and Methods) unless otherwise stated. SPL from uninfected chickens were used as control effectors.

1) Filters

Quantitative filter paper (Whatman No. 542) was used throughout. The filtration of MSB-1 cells labeled with [^{35}S]-methionine through this filter paper under various conditions was

TABLE 1. *Filtration of MSB-1 cells labeled with [^{35}S]-methionine through filter paper (Whatman No. 542) under various conditions*

Expt. No.	[^{35}S]-CPM of MSB-1 cells remaining on the filter paper after washing						% of [^{35}S]-CPM recovered		
	Xo[a]	X_{36}[b]	Xs[c]	A[d]	B[e]	$\frac{Xo-X_{36}}{Xo}\times100$[f]	$\frac{X_{36}-Xs}{X_{36}}\times100$	$\frac{A-B}{A}\times100$[g]
1	15,706± 688[h]	13,538± 572	NT[i]	NT	NT	13.8	—	—
2	35,407±1,038	31,971± 225	4,581±253	NT	NT	9.7	85.7	—
3	34,808± 457	30,516±1,336	2,095±204	NT	NT	12.3	93.1	—
4	NT	34,960±1,019	3,311±144	NT	NT	—	90.5	—
5	NT	17,744± 153	1,709±107	NT	NT	—	90.4	—
6	NT	2,388± 71	278± 14	2,432± 58	1,886± 16	—	88.4	22.5
7	NT	29,101± 373	2,976±110	29,302±919	20,959±204	—	89.8	28.5
Average						11.9	89.7	—

[a] Xo: MSB-1 cells labeld with [^{35}S]-methionine before incubation. [b] X_{36}: MSB-1 cells incubated for 36 hr without effector cells. [c] Xs: Sonicated MSB-1 cells. [d] A: MSB-1 cells incubated for 36 hr with SPL from uninfected chickens. [e] B: MSB-1 cells incubated for 36 hr with SPL from HVT-infected chickens. [f] Spontaneous release of [^{35}S]. [g] Specific cytotoxicity (This formula is described in the Materials and Methods): The test was done at an effector(SPL)-target(MSB-1) cell ratio of 50:1 at 41 C under closed conditions. [h] Average ± standard error of the mean. [i] Not tested.

examined by counting residual radioactivity on the filter paper (Table 1). In the standard procedure for the CMC test without effector cells, the filter retained about 80% of intact MSB-1 cells, but only about 6% of MSB-1 cells which had been damaged by sonication.

2) Atmosphere during incubation

CMC tests were carried out under two different conditions: One test was carried out on Microtest plates under an atmosphere of 5% CO_2 in air; the other test was carried out in a tightly stoppered test tube in a water bath with gentle shaking. As shown in Table 2, the specific [^{35}S]-release in the latter test was higher than that in the former. Therefore, closed test tubes were used in the standard procedure.

3) E/T ratio

The specific [^{35}S]-releases with various ratios of SPL to MSB-1 cells are shown in Fig. 1. Specific activity was evident at a ratio of 25: 1 and was maximal at a ratio of 50: 1 or 75: 1. With further increase in the E/T ratio (100: 1) the specific activity decreased. Therefore, a ratio of 50: 1 was used in the standard microcytotoxicity test.

4) Target cell concentration

Specific [^{35}S]-release at various concentrations of MSB-1 cells per tube is plotted in Fig. 2. The maximum activity was obtained with 5×10^4 MSB-1 cells per 200 μl per tube.

5) Incubation period

The specific [^{35}S]-release after various incubation periods is plotted in Fig. 3. Definite activity was detected after incubation of a mixture of target cells and effector cells for 36 hr. An incubation period of 36 hr seems to be one of the most important factors for detection of activity.

TABLE 2. *Effects of atmosphere during incubation in the CMC test upon specific cytotoxicity*

Condition	Specific cytotoxicity (%)[a]
5% CO_2 in air	21.1
Closed (With shaking)	26.9

[a] Tests were done at an effector (SPL)-target (MSB-1) cell ratio of 50: 1 for 36 hr at 41 C.

2. *CMC test*

The CMC test with SPL and PBL from chickens inoculated with HVT against MSB-1 target cells was investigated. Chickens were inoculated at 2 weeks of age and 2, 4, 6, 8 and

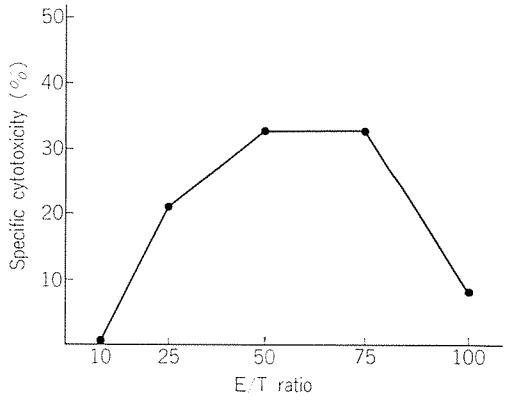

FIGURE 1. Optimum ratio of effector cells (SPL) to target cells (MSB-1) for maximum specific release of [^{35}S]. Tests were done for 36 hr at 41 C under closed conditions.

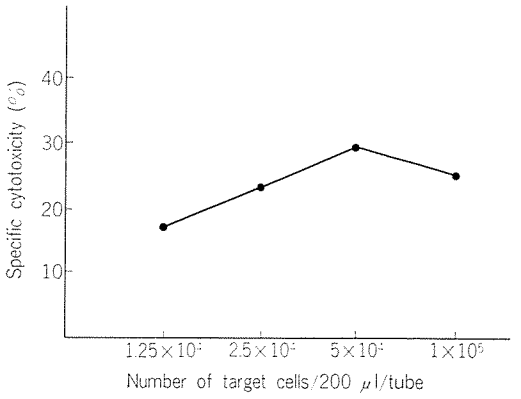

FIGURE 2. Optimum target (MSB-1) cell concentration (at E/T ratio of 50: 1) for maximum specific release of [^{35}S]. Tests were done for 36 hr at 41 C under closed conditions.

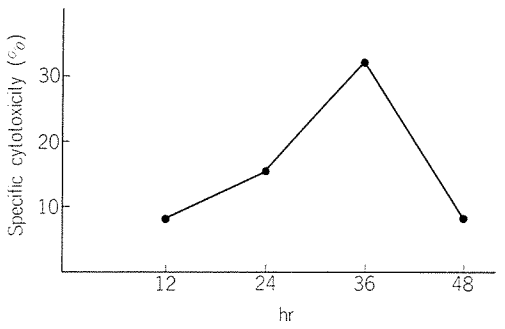

FIGURE 3. Effect of incubation period. Tests were done at an E/T ratio of 50: 1 at 41 C under closed conditions.

10 weeks after inoculation 3 chickens were examined. As controls, SPL and PBL from an equal number of uninoculated broodmates, and, in some experiments, from broodmates inoculated with uninfected CEF were used. The routine procedure for the [^{35}S]-methionine labeling technique, based on the optimum standard conditions described above, was used. Three experiments were carried out and results are summarized in Table 3. Both SPL and PBL from HVT-infected chickens had cytotoxic effects on MSB-1 cells. The cytotoxic effect was marked, especially 4 and 6 weeks after inoculation. On the other hand, no cytotoxic activity against 1104-B cells was demonstrated.

3. *Inhibition of cytotoxicity by anti-T serum*

Effector cell preparations from 3 chickens of 4 to 6-week-old after HVT inoculation were tested. The specificity of the anti-T and anti-B sera used for pretreatment of effector cells were examined by the trypan blue dye exclusion test with thymus and bursa cells from 2-week-old chickens as target cells. As shown in Fig. 4, anti-T serum absorbed with bursa cells and anti-B serum absorbed with thymus cells were toxic in the presence of guinea pig complement to thymus cells and bursa cells, respectively.

Samples of 2.5×10^7/ml of SPL and PBL from HVT-infected chickens and uninfected control chickens were treated with various sera (1: 10 final dilution) and guinea pig complement (1: 10 final dilution) and then the cells were treated with [^{35}S]-methionine labeled MSB-1 cells in the CMC test. As shown in Table 4, the cytotoxicity was almost completely inhibited by treatment of effector cells

TABLE 3. *Cell-mediated cytotoxic response of effector cells from chickens infected with HVT against target cells (MSB-1 and 1104-B) labeled with [^{35}S]-methionine*[a]

Weeks[b] after inoculation	Specific cytotoxicity (%)							
	MSB-1						1104-B	
	Expt. 1		Expt. 2		Expt. 3			
	SPL	PBL	SPL	PBL	SPL	PBL	SPL	PBL
2	7.8	17.8	18.7	12.6	22.8	8.6	3.9	NT[c]
4	18.1	39.1	17.8	31.9 (30.4)[d]	30.6 (24.3)[d]	41.1 (36.3)[d]	6.1	9.0
6	18.0	33.1	23.1 (23.2)[d]	32.3 (27.0)[d]	20.0 (17.4)[d]	42.8 (29.6)[d]	1.9	0
8	4.2	5.2	1.5	9.1	NT	NT	NT	NT
10	0	3.1	NT	NT	NT	NT	NT	NT

[a] Tests were done under the optimum conditions as shown in Table 2 and Figs. 1, 2, 3.
[b] Chickens were inoculated at 2 weeks of age (5,000 PFU/0.2 ml/chicken, i.p.).
[c] Not tested.
[d] Effector cells from chickens stimulated with CEF were used as controls.

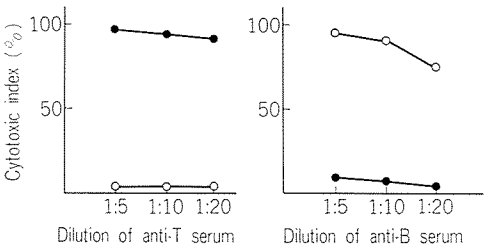

FIGURE 4. Specificities of anti-T and anti-B sera against homologous and heterologous target cells: chicken thymus cells (●——●) and bursa cells (○——○). The target cells were treated with dilutions of antisera in the presence of guinea pig complement. Cytotoxic specificity was estimated by the 0.4% trypan blue dye exclusion test.

with anti-T serum. Pretreatment of the effector cells with anti-B serum also inhibited the cytotoxicity, but not so much as pretreatment with anti-T serum. Treatment of effector cells with normal rabbit serum or complement alone had little effect on the cytotoxicity.

4. *Increase in the cytotoxic activity using purified T cells*

SPL and PBL were fractionated on a Sephadex G-200 immunoabsorbent column bound with anti-B serum. The cells that passed through the column without being adsorbed were >95% T cells, as judged by the

TABLE 4. *Inhibition of cytotoxicity by pretreatment of effector cells with anti-T serum*

Treatment[a]	Specific cytotoxicity (%)					
	SPL			PBL		
	Expt. No.			Expt. No.		
	1	2	3	1	2	3
Anti-T serum	0	2.7	0	0	0.7	2.7
Anti-B serum	4.5	17.3	7.3	6.0	8.5	27.4
Normal rabbit serum	8.5	28.5	23.2	10.0	13.6	19.0
Guinea pig complement alone	10.0	14.0	22.0	9.1	20.4	27.0
None	9.9	21.4	23.1	8.7	31.9	32.3

[a] Effector cells (SPL and PBL) were treated with various sera and guinea pig complement and then treated with [^{35}S]-methionine labeled MSB-1 cells.

TABLE 5. *Increase in cytotoxicity using purified T cells*[a]

Source of effector cells	Specific cytotoxicity (%)			
	MSB-1		1104-B	
	Expt. No.		Expt. No.	
	1	2	1	2
Untreated SPL	14.1	7.8	6.0	NT
T cells purified from SPL	40.2	15.1	0	0
Untreated PBL	31.8	17.8	9.0	NT
T cells purified from PBL	42.4	21.6	8.3	NT

[a] T cells were purified from SPL and PBL by affinity chromatography and then treated with [^{35}S]-methionine labeled target cells.

indirect IF test. As shown in Table 5, the cytotoxic activity of these T cells in the CMC test was greater than those with the original SPL and PBL.

5. *Possible involvement of antibody-dependent cell-mediated cytotoxicity (ADCC) activity in the CMC test*

Three 2-week-old chickens were vaccinated with 5,000 PFU of HVT. Four weeks later the antibody titer of their pooled sera to HVT-infected cells was >512, estimated by the indirect IF test. Volumes of 0.1 ml of serial dilutions of the anti-HVT serum per tube were added to mixtures of PBL from 3 uninfected broodmates and [^{35}S]-methionine labeled MSB-1 cells and the specific cytotoxicity was examined by the standard procedure for the CMC test. Two experiments were carried out. As a control, pooled sera from 3 uninfected broodmates was used in place of anti-HVT serum. As shown in Table 6, no significant cytotoxicity was demonstrated with any dilution of the sera. Thus the cytotoxicity shown in Table 3 does not seem to be due to ADCC activity by the sera of HVT-vaccinated chickens.

TABLE 6. *Possible involvement of ADCC activity in the CMC test*

Dilution of anti-HVT serum	Specific cytotoxicity (%)	
	Expt. 1	Expt. 2
1 : 30	0	0
1 : 300	0	0.4
1 : 3,000	0	4.0

6. *CDAC test*

The CDAC activity of serum of HVT-vaccinated chickens against MSB-1 cells was examined using duck complement. Chicken anti-MSB-1 serum was used as a positive control. As shown in Fig. 5. serum of HVT-vaccinated chickens was not cytotoxic to MSB-1 cells, but chicken anti-MSB-1 serum was cytotoxic to MSB-1 cells. Neither of these sera showed any significant cytotoxicity against 1104-B cells.

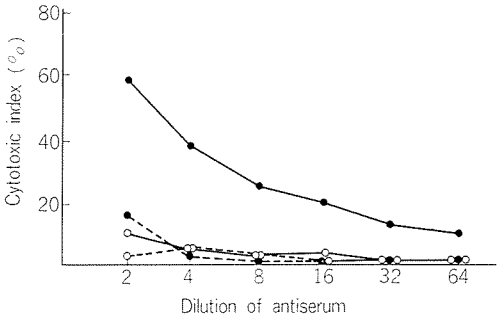

FIGURE 5. CDAC tests of chicken anti-HVT serum (○) and anti-MSB-1 serum (●) against MSB-1 cells (——) and 1104-B cells (·····) labeled with [^{35}S]-methionine. Target cells were treated with serial dilutions of antisera in the presence of duck complement. Target cells mixed with serial dilutions of antisera without complement were used as controls.

DISCUSSION

There are several reports on the cytotoxicity of sensitized lymphocytes from chickens vaccinated with HVT on MD lymphoma line cells (Powell, 1976; Sharma, 1977b; Sugimoto et al., 1978). Sharma observed cytotoxicity but the other authors failed to detect it. In this work, we found that both SPL and PBL from HVT-vaccinated chickens were cytotoxic to MSB-1 line cells in the CMC test by the [^{35}S]-methionine labeling technique. Thus the discrepancy in previous results may be due to differences of experimental conditions, such as differences in techniques, incubation periods of the target-effector cell mixture, and the age of chickens vaccinated with HVT. The [^{35}S]-methionine labeling technique that we used is similar to the techniques of labeling with [^{3}H]-thymidine (Hartzman et al., 1972), [^{3}H]-uridine (Hashimoto and Sudo, 1971) or [^{3}H]-proline (Bean et al., 1973; Dambrine et al., 1977). Dambrine et al. (1977) labeled MSB-1 cells with [^{3}H]-proline for 20 hr.

However, 3 hr was sufficient time to label MSB-1 cells with [^{35}S]-methionine, because of the high specific radioactivity of the methionine used. Techniques using radioactive amino acids have an advantage over that using [^{51}Cr] in that less spontaneous release of isotope occurs during the incubation period in the CMC test. Thus use of [^{35}S]-methionine was useful in this work, since 36 hr was the optimum incubation period in our CMC test, and so we had to avoid spontaneous release of a large amount of isotope. Use of a 36 hr incubation period seems to be one of the main reasons why we could clearly detect cytotoxicity in the CMC test. The use of filter papers that allowed passage of most of the damaged cells but retained most of intact cells also helped in obtaining clear results.

The cytotoxicity was inhibited by pretreatment of the effector cells with anti-T serum and complement, and was increased by use of purified T cells. The involvement of ADCC activity possibly present in the anti-HVT serum in the CMC test could be excluded, because anti-HVT serum did not show any ADCC activity against MSB-1 cells. All these results indicate that T cells are important for effector activity in the CMC test.

In mice (and also in rats, humans and chickens) immune effector function mediated by T cells seems to be restricted by the major histocompatibility gene complex (Zinkernagel et al., 1978). Virus-specific cytotoxic T cells generated in mice infected with lymphocytic choriomeningitis virus (Zinkernagel and Doherty, 1974; Doherty and Zinkernagel, 1975) lyse infected target cells only when both are compatible at H-2K or H-2D of the H-2 gene region. Since the chickens used in our experiments were not inbred, they probably shared a histocompatibility antigen of the B locus distinct from that of MSB-1 cells. The dependency of CMC activity on the major histocompatibility antigen of effector and target cells in our system remains unknown. Further investigations are required using an MD lymphoma cell line such as MOGA-1 or 2 (Yamaguchi et al., 1979) with a known major histocompatibility antigen.

CMC activity seemed to be specific to MSB-1 cells, because effector cells toxic to MSB-1 cells did not react with 1104-B cells. Most (more than 95%) MSB-1 cells express MATSA (Witter et al., 1975; Matsuda et al., 1976b), but few (less than 1%) express MDV-induced cell surface antigen (Akiyama and Kato, 1974). 1104-B cells, a B cell-derived lymphoblastoid cell line (Matsuda et al., 1976a) productively infected with avian leukosis virus, do not show any MDV-coded expressions, including MATSA. It is conceivable that the cytotoxic activity described above could be directed against MATSA of MSB-1 cells. However, the mechanism of induction of anti-MATSA immunity in chickens vaccinated with HVT remains unknown, because HVT has not been shown to have any ability to transform chicken lymphocytes or to express MATSA. We are now attempting to find MATSA positive cells in HVT-vaccinated chickens.

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