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Cell-Mediated Immunity to Tumor Antigen in Marek's Disease: Susceptibility of Effector Cells to Antithymocyte Serum and Enhancement of Cytotoxic Activity by *Vibrio cholerae* Neuraminidase

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Spleen cells from chickens inoculated 7 to 8 days previously with Marek's disease virus were cytotoxic for ^{51}Cr-labeled cells of a Marek's disease lymphoblastoid cell line (MSB-1 line) in a 4-h in vitro cytotoxic assay. The cytotoxic activity of spleen cells was inhibited by pretreatment with antithymocyte serum and complement, but not with complement alone or in combination with antibursa cell serum or normal preimmune serum. The conclusion was that the effector cell in the above cytotoxic assay was a thymus-derived lymphocyte. Also, pretreatment of target cells with *Vibrio cholerae* neuraminidase enhanced in vitro cytotoxic activity of effector cells. Similar enzymatic treatment of effector cells had a negligible effect on cytotoxicity.

Marek's disease virus (MDV) is a herpesvirus with documented potential for oncogenesis in chickens. The virus transforms T lymphocytes and induces in transformed cells a tumor antigen designated MATSA, an acronym for Marek's disease tumor-associated surface antigen (34). The recognition of MATSA as a marker for cellular transformation by MDV has generated interest in the possible role this antigen may play in the pathogenesis of the disease, particularly in natural or vaccine-induced resistance to Marek's disease (MD).

Recently, I and others have found that chickens inoculated with MDV develop a cell-mediated immune response to MATSA (20, 27). This response apparently develops against transformed cells generated in vivo by MDV.

The response was detected in an in vitro cytotoxicity test by reacting effector cells from virus-infected chickens with ^{51}Cr-labeled target cells obtained from cell lines derived from MD lymphomas. All cell lines developed thus far from MD lymphomas consist of T lymphoblasts that carry the MDV genome and MATSA and are thus considered transformed by MDV. In virus-infected chickens, the cytotoxic effector cells have been detected in the spleen (27) as well as in the peripheral circulation (20). Generally the levels of cytotoxic activity detectable in in vitro assays have been quite low. The nature of effector cells and the characteristics of their activity are not known.

The main objective in this study was to identify the cytotoxic cell and to determine whether cytotoxic activity of effector cells could be influenced by pretreatment of target cells with neuraminidase. Similar modification of target cells has been shown to increase the expression of tumor antigens and thus augment the immune response against them (7, 24).

MATERIALS AND METHODS

Animals. Chickens were line 7 or F_1 progeny of a cross between lines 7 and 15 being maintained at this laboratory (33). The breeder flocks were held under specific-pathogen-free conditions and were free from natural infection with common poultry pathogens including herpesvirus of turkeys and MDV. All experimental chickens were held in Horsfal-Bauer-type cages under negative pressure from the day of hatch through the duration of the experiment.

New Zealand white rabbits were young adults obtained from a commercial source.

MDV. The JM strain of MDV was cloned as follows. Cell-free virus, extracted from the skin of MDV-infected line 7 chickens, was propagated in chicken kidney cell cultures under agar overlay. An individual, well-separated plaque was picked, and the progeny virus from this plaque was inoculated into 1-day-old chickens. Three weeks later, cell-free virus obtained from inoculated chickens was cloned again in chicken kidney cells. After a third similar cloning step, the virus was propagated in duck embryo fibroblast cells and stored at −196°C as cell-associated virus stock. This stock, designated clone 111S, had undergone 11 tissue culture passages before use in this study.

Effector cell preparation. Chickens were inoculated with 10^3 plaque-forming units of MDV at 6 days

of age. At the peak of the cytotoxic response (27), 7 to 8 days after inoculation, three to five infected and usually an equivalent number of uninfected control chickens were sampled, and their spleens were removed aseptically. Single spleen cell suspensions were made as described earlier (27), except that the cell suspensions were clarified by being layered on a Ficoll-Hypaque gradient at specific gravity 1.09 and centrifuged for 10 min at 300 × *g*. Viable cells recovered from the interphase were used as effector cells. Surviving chickens from infected and uninfected control groups were raised under isolation for 3 to 4 weeks and were monitored for infection with MDV either by testing their sera for MDV antibody or by examining histological sections of peripheral nerves and gonads for lesions of MD. Infection with MDV could be consistently demonstrated in the inoculated groups but not in control groups.

Target cells. The cells of the MSB-1 line were used as targets. The MSB-1 line is a lymphoblastoid cell line developed by Akiyama and Kato (1) by propagating in vitro cells of a spleen lymphoma from a chicken affected with MD. The MSB-1 cells contain MATSA (34) and grow as suspension cultures as described in detail (1, 17). For this study, we used cells from a single frozen stock held at −196°C (27). Rapidly growing cells within 24 h of subculture were labeled with 100 mCi of $Na_2{}^{51}CrO_4$ (specific activity, 229 mCi/mg; concentration, 1 mCi/ml; New England Nuclear, Boston, Mass.) for 45 min at 37°C according to the procedures described in detail earlier (27), except that at the end of the labeling period, cells were washed and used without further centrifugation on bovine serum albumin gradient.

In one experiment, cells of the TLT line were used as target cells. This line, provided by K. Nazerian, was derived from a lymphoid leukosis tumor in an outbred white leghorn chicken (32). The TLT cells are lymphoblastoid in nature; they lack MATSA and are productively infected with ribonucleic acid tumor virus.

Cell-mediated cytotoxicity assay. The general procedure was as described previously (27). Briefly, 0.1 ml of target cell suspension containing 10^5 ^{51}Cr-labeled MSB-1 cells was mixed with 0.1 ml of effector cell suspension containing 5×10^6 spleen cells (effector/target ratio of 50:1) in Microtest plates (3040 Microtest II, Falcon Plastics, Oxnard, Calif.) and incubated for 4 h at 37°C in a 3 to 5% CO_2 atmosphere. Two or three replicate wells were used for each effector cell preparation. At the end of 4 h the amount of ^{51}Cr released into the medium and total and spontaneous release were measured as described (27). The percentage of specific release of ^{51}Cr was calculated by the following formula:

$$\% \text{ specific release} = \frac{\text{cpm in target cells mixed with infected spleen cells} - \text{cpm in target cells mixed with normal spleen cells}}{\text{cpm in target cells mixed with detergent [maximum release]} - \text{cpm in target cells mixed with normal spleen cells}} \times 100$$

Complement-dependent antibody cytotoxicity assay. This assay was conducted in plastic tubes (16 by 125 mm; Falcon Plastics). Sera were diluted in the test medium (RPMI + 5% fetal bovine serum + 0.1 M HEPES [*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid] buffer + 100 U of penicillin and 100 μg of streptomycin per ml). Each sample was tested in duplicate tubes. A 0.1-ml portion of serum dilution was mixed with 0.1 ml of target cell suspension containing 2×10^5 ^{51}Cr-labeled target cells. To this mixture was added 0.2 ml of an appropriate dilution (in Veronal buffer) of guinea pig complement (Difco Laboratories, Detroit, Mich., control no. 632753). The lyophilized complement was reconstituted according to the manufacturer's directions and absorbed once for 30 min at 4°C with approximately 1/20 (vol/vol) of normal chicken lymphocytes. The optimum concentration of complement was predetermined in a homologous antigen-antibody reaction in the ^{51}Cr release assay.

The target cell-antibody-complement mixture was held at 37°C for 45 to 60 min. At the end of incubation, 1.1 ml of cold Dulbecco phosphate-buffered saline (1.1× concentration containing 2% fetal bovine serum and 100 U of penicillin and 100 μg of streptomycin per ml, adjusted to pH 7.4 with 1 N NaOH) was added to each tube. The tubes were centrifuged at 700 × *g* for 5 min, and 1.0-ml portions of the supernatant fluid were carefully removed without disturbing the pellet and counted for gamma emission in a Beckman 300 gamma counter (Beckman Instruments Inc., Irvine, Calif). Counts per minute were averaged for each sample. Controls in each test consisted of target cells mixed with (i) preimmune serum and complement; (ii) complement without serum; (iii) medium alone (spontaneous release); and (iv) Triton X-100 detergent (maximum release). Generally, less than 10% of the total releasable isotope was recovered in the medium in controls (i), (ii), and (iii) above. The percentage of specific release was determined as follows:

$$\% \text{ specific release} = \frac{\text{cpm in test sample in complement control}}{\text{maximum releasable cpm} - \text{cpm in complement control}} \times 100$$

Antisera. Antithymocyte and anti-bursa cell sera were prepared by a similar procedure; two rabbits per cell type were used. Preimmunization serum samples from rabbits lacked anti-complementary activity. Thymus and bursa tissues were removed from 3-week-old chickens of line 7_2. The age of donor chickens was considered critical because at 3 weeks, cross-contamination of thymus and bursa is minimal (2, 12). Single-cell suspensions prepared as described earlier (27), containing 0.5×10^8 to 1×10^8 cells, were injected intravenously in each rabbit. Two additional similar biweekly intravenous inoculations were given. Rabbits were bled 7 to 10 days after the last inoculation, and sera were repeatedly cross-absorbed with thymus or bursa cells from 3-week-old line 7_2 donors until all

cross-reactivity was reduced to background levels.

Treatment of effector cells with antisera. Effector cells (10×10^6) were treated with a 1:4 final dilution of antiserum for 30 min at 37°C. The cells were then washed once, resuspended in 0.1 ml of medium, and incubated at 37°C for an additional 30 min with an equal volume of an optimum dilution of complement. After three washes, the cells were used in the cytotoxicity assay at an effector/target cell ratio of 50:1.

Neuraminidase. The *Vibrio cholerae* neuraminidase (VCN, B grade, lot 600786; activity, 500 U/ml; Calbiochem, San Diego, Calif.) was used. Cells (10×10^6) suspended in serum-free Dulbecco phosphate-buffered saline were treated with various concentrations of the enzyme for 30 min at 37°C. After treatment, the cells were washed three times before use in the cytotoxicity assay.

The Student *t* test was used to analyze data.

RESULTS

Specificity of antithymocyte and antibursa cell serum. Reactivity of absorbed sera against homologous and heterologous cells is shown in Fig. 1. The target cells were ^{51}Cr-labeled thymus or bursa cell suspensions of 3-week-old line 7 donors. Both sera had appreciable specific activity with minimal (less than 10% release) reactivity against the heterologous cell type.

Inhibition of cytotoxicity by antithymocyte serum. Effector cell preparations from four chickens at the peak of cytotoxic activity (8 days after MDV inoculation) were tested (Table 1). Three chickens had appreciable cytotoxic levels. The cytotoxicity in all four preparations was almost completely inhibited by pretreatment of effector cells with antithymocyte serum. Pretreatment of effector cells with antibursa cell serum, normal rabbit serum, or complement alone had a negligible effect on cytotoxicity. The results indicated that the effector cells shared antigenic determinants with thymus cells.

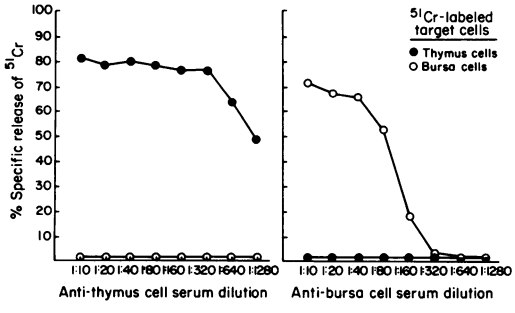

FIG. 1. *Reactivity of anti-thymus cell and anti-bursa cell sera against homologous and heterologous target cells. The target cells were labeled with ^{51}Cr and reacted with the dilutions of antisera indicated in the presence of guinea pig complement.*

TABLE 1. *Inhibition of cytotoxicity by pretreatment of effector cells with anti-thymus cell serum*

Treatment[a]	% Specific release of ^{51}Cr by effector cell prepn no.:			
	1	2	3	4
Anti-thymus cell serum	0	1.6	0.9	0.4
Anti-bursa cell serum	3.2	8.9	7.7	7.4
Preimmune rabbit serum	2.8	9.5	9.2	8.0
Guinea pig complement	2.8	9.8	9.6	8.2
None	4.0	9.0	9.7	9.8

[a] 10×10^6 effector cells (spleen cells from MDV-infected chickens) were treated with various sera and complement before the cells were reacted with ^{51}Cr-labeled MSB-1 target cells in a 4-h cytotoxicity test.

Effect of VCN. The effect of VCN treatment of MSB-1 cells was studied in two experiments (Table 2). The MSB-1 target cells were treated with various concentrations of VCN and then labeled with ^{51}Cr and reacted against effector cells obtained from chickens inoculated 7 to 8 days previously with MDV. In experiment 1, target cells treated with each concentration of VCN were reacted with effector cell preparations from two chickens. The specific release of ^{51}Cr from untreated target cells ranged from 6.1 to 9.4%. This activity was enhanced if target cells were pretreated with ≥100 U of VCN ($P < 0.05$, *t* test). Maximum enhancement of cytotoxic activity was noted with 150 U of VCN.

In experiment 2 (Table 2), effector cell preparations from seven chickens were examined. Each preparation was reacted simultaneously with target cells pretreated with varying concentrations of VCN. The cytotoxic activity against untreated target cells ranged from 5.7 to 30.4%. This activity could be enhanced by pretreating target cells with VCN, although the level of enhancement by different preparations of effector cells varied widely. In experiment 2, the average increase in cytotoxic activity by using VCN-treated target cells was significant ($P < 0.01$). All doses of VCN used enhanced cytotoxicity, and differences in enhancement levels between doses of VCN were not significant ($P > 0.05$), although, as in experiment 1, maximum enhancement occurred with 150 U of VCN.

Treatment of MSB-1 cells with VCN did not alter the specificity of the reaction because immune cells that expressed enhanced cytotoxicity against VCN-treated MSB-1 cells were not cytotoxic for VCN-treated target cells of the MATSA-lacking TLT line (Table 3). Treatment of MSB-1 cells with a heat-inactivated preparation of VCN did not enhance cytotoxicity. Furthermore, spleen cells from a normal control

TABLE 2. *Enhancement of cytotoxic activity against MSB-1 cells pretreated with VCN*

Expt	Chicken no.	% Specific release of ^{51}Cr from:					
		Untreated target cells	Target cells treated with VCN				
			50[a]	100	150	200	250
1	1	6.9	6.0				
	2	6.5	5.2				
	3	6.9		10.2			
	4	7.2		11.5			
	5	6.9			14.3		
	6	7.2			14.3		
	7	9.4					11.7
	8	6.1					9.5
2	1	25.5	35.5	34.0	31.0	38.6	
	2	30.4	38.6	37.1	33.4	36.4	
	3	14.6	21.8	23.1	23.6	23.8	
	4	8.8	10.6	12.1	20.3	9.1	
	5	5.7	9.0	10.0	11.6	9.0	
	6	25.4	37.1	34.3	39.7	40.9	
	7	17.2	20.1	22.7	19.9	22.6	
1 + 2[b]			24.8	45.5	67.2	41.1	40.1

[a] Units of VCN per 10×10^6 cells.

[b] Data indicate average percentage of increase (average of both experiments) using VCN-treated target cells.

TABLE 3. *Specificity of enhanced cytotoxic activity against VCN-treated MATSA-bearing target cells*

Source of effector cells	Treatment of target cells[a]	% Release of ^{51}Cr from target cells[b]	
		MSB-1	TLT
Immune spleen	None	17.0	7.4
	VCN	23.2	9.8
	IVCN	17.0	8.9
Normal spleen	None	8.9	7.1
	VCN	9.2	7.4
	IVCN	8.9	6.7

[a] 5×10^6 to 10×10^6 target cells were treated with medium (none), 100 U of VCN (VCN), or 100 U of VCN inactivated at 70°C for 1 h (IVCN).

[b] Calculated by dividing counts per minute released in the effector cells and target cell mixture (50:1) by total releasable counts per minute and multiplying by 100.

donor were not cytotoxic for VCN-treated MSB-1 cells.

The results noted above indicated that pretreatment of MSB-1 target cells with VCN enhanced cytotoxic activity of MD-immune effector cells. However, treatment of effector cells with various concentrations of VCN before their reaction with untreated target cells did not affect cytotoxic levels (Table 4).

DISCUSSION

The process of tumorigenesis in MD is not entirely clear. Because a certain proportion of lymphoma cells contain MATSA (21, 34), lymphomas are most likely initiated by cellular transformation by MDV. The transformed foci may recruit other, normal lymphoid cells that may possibly engage in immunological reactions against the transformed cells.

The identity of the transformed cells in vivo is not unequivocally proved, although circumstantial evidence strongly suggests that the transformed fraction in the lymphoma is a T lymphocyte. T cells form a clear majority in MD lymphomas (14, 23, 28), and, most important, all in vitro propagating cell lines derived from MD lymphomas consist of T lymphoblasts (16, 21). Also, we have recently obtained evidence that T cells may serve as target cells in MD lymphoma formation (28). We noted that under experimental conditions, chickens made severely deficient in T-cell population by surgical thymectomy, accompanied by total-body gamma irradiation and repeated injections with antithymocyte serum, developed significantly fewer gross lymphomas than did T-cell-intact hatchmates. B-cell depletion accompanied by agammaglobulinemia had been previously shown to have no effect on MD (11, 18). Because T cells apparently transform under the influence of MDV, it is of interest that in the present study the effector cells involved in cell-mediated immunity in MD were also the T lymphocytes. Assuming that the cytotoxic response to MATSA participates in immune surveillance against MD (19), it seems somewhat paradoxical that the same cells would be involved in such opposing functions, i.e., target cells for transformation and effector cells for cytotoxicity against the transformed cells.

The apparent functional diversity of T cells in MD may be explained if different subpopulations of T cells are involved. Subpopulations within T cells have been well recognized in certain mammalian systems (5, 15). Studies by Shiku et al. (31) and by Cantor and Boyse (3, 4) clearly link distinctive Ly surface antigenic markers on T cells with different immunological functions. The in vitro killer cell activity of nonadherent immune peritoneal exudate cells was primarily associated with T cells with Ly-

TABLE 4. *Effect of VCN treatment of effector cells on cytotoxic activity*

Effector cell prepn no.	% Specific release of ^{51}Cr by effector cells treated with:			
	0 U of VCN	50 U of VCN	100 U of VCN	150 U of VCN
1	9.4	10.3	10.6	9.7
2	3.2	3.1	2.4	2.7
3	14.7	16.5	15.3	16.4

2,3 surface markers and not with those with Ly-1 markers (31). Conversely, helper activity in development of antibody response to T-cell-dependent antigens was primarily the function of Ly-1 and not of Ly-2,3 cells (3). In mixed-lymphocyte cultures, the cells that developed killer cell activity against alloantigens also belonged to the Ly-2,3 subclass. The Ly-1 cells markedly enhanced the killer cell activity but did not by themselves serve as killers (4). The avian T-cell system has not been studied comprehensively, although some evidence shows that chicken thymus also contains subpopulations of T cells (9, 35) and that different lineages may be derived from different sources of prethymic stem cells (8). Recently, a distinct alloantigen (designated Th-1) was detected on T cells of two highly inbred lines of chickens (12). Characterization of the T-cell subpopulation in chickens may be helpful in resolving the dual role attributed to T cells in MD.

The recognition of T cells as the effector cells in cell-mediated immunity in MD is consistent with the results obtained previously in immunosuppression studies. Although chickens inoculated with MDV mount a rather vigorous humoral immune response, I and others had found that immunosuppression in the B-cell functions did not directly influence susceptibility or resistance to this disease (11, 18, 26, 29). On the other hand, immunosuppression in the T-cell functions compromised immune surveillance against MD (30). Two-month-old chickens ordinarily resistant to MD developed progressive tumors if they were deficient in delayed-type hypersensitivity response because of neonatal thymectomy and total-body gamma irradiation before MDV exposure.

The levels of cytotoxic activity detectable in in vitro cytoxicity tests in MD have been generally quite low, and in the present study I made an effort to enhance cytotoxic levels by treating target and effector cells with VCN. VCN cleaves linkages between sialic acid and mucopolysaccharides on cell surfaces (10) and may alter the immunogenicity of cells by exposing surface antigens that may not be fully expressed because of sialic acid residues (6, 24). Thus, lymphoid cells treated with VCN become more susceptible to lysis by antibody in vitro (22) and become more antigenic and elicit stronger immune responses in the host than do untreated cells (7, 24). VCN treatment of effector cells may also enhance their activity by exposing receptor sites that interact with the surface antigens present on target cells (13). Our results indicated that spleen cells from MDV-infected chickens produced higher specific release of the isotope from VCN-treated MSB-1 target cells than from untreated target cells. Increased cytotoxic activity was specific to MSB-1 cells, and VCN treatment of effector cells was ineffective in altering cytotoxicity. Enhanced sensitivity of the cytotoxicity test by using VCN-treated target cells may be a useful aid in in vitro studies of cell-mediated immunity in MD.

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