Brief Definitive Report

IgM-INDUCED TUMOR CELL CYTOTOXICITY MEDIATED BY NORMAL THYMOCYTES*

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Adult murine hosts undergoing induction and regression of primary Moloney sarcoma virus (MSV) tumors develop antibodies (1–3) and lymphocytes (4–6) which have activity in vitro against Moloney leukemia virus (MLV)-determined cell surface antigens on the tumor target cells (7). Antibodies which are able to lyse the appropriate virus-induced tumor target cells in the presence of complement appear in two peaks with respect to time after MSV injection (1). The first peak occurs in the early tumor-bearing animals 10–15 days after MSV (1). This is followed by a drop in the antibody titers in early regressors whereafter the antibodies rise and maintain high titers in long-term regressor animals (1, 8). The early as well as the late peaks contained cytotoxic activity in both the IgG and IgM fractions (1). Such MLC-specific antibodies are also active in the induction of tumor cell destruction in vitro by normal lymphoid cells (8). We have recently reported that both IgG and IgM were able to induce cytotoxicity by nonimmune spleen cells as well as potentiate the cytotoxic activity of immune spleen cells (9). We now report that IgM from MSV regressor mice will induce cytotoxicity against the tumor target cells not only by normal spleen cells but by normal thymocytes and the thymus cell cytotoxicity in combination with IgM appears to be more efficient than that produced with spleen cells.

Materials and Methods

Source of Antisera. Adult BALB/c mice injected with 0.1 ml MSV-M (MSV-B-64, courtesy of Dr. Jack Gruber, NCI, Bethesda, Md.) developed tumors in 5–8 days which usually regressed by day 20–25. The serum pooled from 10 such animals at 30 days after MSV injection was heat inactivated at 56°C and stored at −20°C before use. Serum fractionation was carried out on Sephadex G-200 using a

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90 × 1.5 cm column as previously described (1, 9). The first half of the leading protein peak was pooled and reconcentrated to the original serum volume. This fraction was demonstrated to be IgM by the following assays: immunoelectrophoresis of the 19S fraction with rabbit antiserum against whole mouse serum (Hoechst Pharmaceuticals, Inc., Kansas City, Mo.) gave a single band in the IgM region. Furthermore, on immunodiffusion against goat antimouse IgM and goat antimouse IgG (Cappel Laboratories, Inc., Downington, Pa.) the 19S peak reacted only with the heavy-chain-specific anti-IgM.

Cytotoxicity Assays. Serial dilutions of the antiserum or the above fraction were placed on Ha2 target cells (an MLV antigen possessing in vitro cell line (7, 10) derived from an MSV-induced tumor of a CBA mouse) which had been seeded 3–4 h previously in wells of microplates (no. 3034, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at a concentration of 200 cells per well. After 1 h incubation, macrophage-deficient splenocytes or thymocytes prepared as described previously (8), from adult CBA or BALB/c mice were added to the wells at a concentration of 10,000 cells per well. The plates were then incubated for 20 h at 37°C in 5% CO_2. All dilutions and incubations were done in minimal essential medium with Earle's salts containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 50 μg/ml streptomycin. The number of target cells remaining in each well was counted and the log mean ± SD of six replicate wells for each dilution was calculated. Comparisons were made between control wells containing lymphocytes and test wells containing antibody plus the same number of lymphocytes. Antibody alone at the same concentrations was also incubated on the target cells in parallel plates without the addition of lymphocytes as a further control. In order to determine if lysis was being induced as well as reduction of target cells in the microplate assay, a chromium release test was performed. Ha2 target cells were labeled with ^{51}Cr (Na_2 CrO_4, New England Nuclear, Boston, Mass.), 100 μCi/10^6 cells. 2×10^4 labeled target cells were incubated with a 1:320 final dilution of MSV-immune IgM for 1 h followed by the addition of 2×10^6 lymphocytes. After 24 h incubation at 37°C in 5% CO_2, samples of the supernates were assayed in a gamma counter to determine ^{51}Cr release.

Results

The antibody-induced cell-mediated cytotoxicity as assayed in microcytotoxicity tests is shown in Table I. This pool of serum from regressor mice induced 100% cytotoxicity in the presence of normal CBA or BALB/c spleen cells which had been rendered macrophage deficient (8) before the assay. Both types of spleen cells produced this level of cytotoxicity to a serum dilution of 1:40. However, the end point titer using CBA spleen cells was 1:1,280, whereas, it was only 1:80 with BALB/c spleen cells. The IgM fraction of this antiserum also induced cytotoxicity by normal CBA spleen cells. As indicated in the table the maximum cytotoxicity mediated by spleen cells was 34%. This decreased as the twofold dilutions were made to an end point titer of 1:640 at which the target cell reduction was 16%. The IgM induced a somewhat different cytotoxicity pattern with normal thymus cells; maximum reduction of 45% occurred at a dilution of 1:320. The last dilution showing significant target cell reduction was 1:1,280. As indicated in the table, this IgM incubated on the target cells alone produced no significant target cell reduction nor did normal serum from an uninfected mouse in combination with the same number of normal spleen cells.

Results of the chromium release assay are shown in Table II. Using a 1:320 dilution of the IgM fraction, specific chromium release above the level of spontaneous release was observed only with the combination of IgM and normal thymus cells. Spleen cells were not induced to be cytotoxic in this test. This is seemingly in contrast to the microplate assay. However, the optimum dilution of

TABLE I

Antibody-Induced Cell-Mediated Cytotoxicity against Ha2 Target Cells in Microcytotoxicity Assays

Sera*	Lymphocytes‡	Target cells in control§	Maximum reduction‖	Optimal dilution¶	Titer**
		log_{10}/mean ± *SD*	%		
MSV 30 R unfractionated	CBA spleen	2.002 ± 0.07	100	(10-40)	1,280
MSV 30 R unfractionated	BALB/c spleen	1.932 ± 0.06	100	(10-40)	80
MSV 30 R IgM	CBA spleen	1.996 ± 0.05	34	10	640
MSV 30 R IgM	CBA thymus	2.119 ± 0.07	45	320	1,280
MSV 30 R IgM	None	1.873 ± 0.20	—	—	<10
Control serum	BALB/c spleen	2.395 ± 0.06	—	—	<10

* Sera placed on target cells in nine twofold dilution steps beginning at a dilution of 1:10; MSV 30R indicates serum from regressor mice 30 days after MSV injection; control serum was taken from an uninfected BALB/c mouse.

‡ Macrophage-depleted lymphoid cells, 10,000 per well, prepared as described previously (5, 8, 9). Thymocytes contained <2% B cells and >95% T cells by surface Ig and rabbit anti-T-cell (8) serum cytotoxicity, respectively.

§ Control contained lymphocytes alone or medium alone as indicated. Log_{10} mean ± SD calculated from six replicates.

‖ % reduction calculated by the formula: 100 − (geometric mean of target cells in test wells)/(geometric mean of target cells in control wells) × 100. Numbers shown indicate maximum observed. (—), indicates no significant reduction.

¶ Optimal dilution, numbers shown indicate the reciprocal of the dilution(s) where the maximum reduction occurred. *P* values for each of these were <0.0005 except the last two indicated by (—) where no significant reduction occurred.

** Reciprocal of the end point dilution producing significant ($P < 0.05$) target cell reduction.

TABLE II

IgM-Induced Thymocyte-Mediated ^{51}Cr Release from Labeled Ha2 Cells

Ha2 target cells incubated with*	Mean ± SD of cpm released‡	cpm Above spontaneous release‡	*P*§
Medium	1,177 ± 95	—	
IgM	1,163 ± 112	—	—
IgM + thymocytes	1,467 ± 80	289	<0.01
Thymocytes	1,113 ± 12	—	—
IgM + splenocytes	983 ± 104	—	—
Splenocytes	1,213 ± 14	36	>0.25

* IgM from MSV regressor mice 30 days after infection diluted 1:320.

‡ Mean values from triplicate samples.

§ *P* values derived using a Student's *t* test to compare the triplicate values for spontaneous release in the medium control with the combinations shown. Only the IgM plus thymocyte combination produced ^{51}Cr release significantly greater than spontaneous release.

IgM for cytotoxicity by spleen cells was much lower than it was with thymus cells. As indicated, there was no induction of lysis with either IgM alone or normal thymus cells alone.

Discussion

We have thus demonstrated the ability of IgM antibody from MSV regressor mice to induce cytotoxicity by normal thymus and spleen cells against target cells bearing the appropriate virally induced antigen(s). In addition to the specificity controls reported herein, it has been demonstrated repeatedly in our hands that the antibody and lymphocyte cytotoxicity in this system is specific for

MLV-determined cell surface antigen(s) (1, 5, 7, 8). Antibody from MSV-infected hosts will react specifically with MLV-induced lymphoma cells (1) and with cells which possess both MSV and MLV (S+ L+ cells) (7, 8) such as the Ha2 cells used in the present experiments, but not with cells which possess only the defective sarcoma virus and are leukemia negative (S+ L− cells) (7). The target cell specificity of the immune lymphocytes in this system has also been repeatedly demonstrated (5, 7, 8, 11–13). Finally, we have also demonstrated the target cell specificity of the antibody-dependent cell-mediated cytotoxicity to be specific for cells possessing MLV-determined cell surface antigen(s) (14).

In many nontumor systems which have been investigated, it has been reported that IgG induced antibody-dependent cell-mediated cytotoxicity and that IgM is not active in such systems (15–17). However, we have found that IgM is capable of inducing cytotoxicity against target cells bearing the appropriate antigenic specificities in vitro by normal lymphoid cells as well as potentiating the cytotoxic activity of immune lymphoid cells (9). Previous investigations, including those by ourselves, had indicated that thymus-processed lymphocytes (T cells) were not required for antibody-dependent cell-mediated cytotoxicity (8, 18, 19) and furthermore, that normal T-cell splenic lymphocytes did not appear to be active in such systems (8). There has been one report, however, indicating that antibody could induce cytotoxicity by normal rabbit thymus cells (20). We are currently investigating the subpopulations of thymus cells and spleen cells which can be induced to be cytotoxic by IgM and IgG with specificity for the virally induced antigens on the surface of the tumor target cells. Although the in vivo significance of the present findings is unknown, this IgM-induced thymocyte cytotoxicity may be one of the factors which produce the high incidence of spontaneous regression of these primary MSV tumors.

In addition to the implications in this particular tumor model, these findings may have a broader significance in terms of cellular immunity in general. Since T cells, which by themselves are not cytotoxic, can be induced to be cytotoxic against target cells coated with IgM, it is conceivable in other situations of T-cell-dependent in vitro cytotoxicity that IgM with specificity for the target cells is involved. IgM secreted by bone marrow-derived bursa equivalent-processed lymphocyte possessing high concentrations of surface Ig (B cells) could bind to the target cells and induce or potentiate the cytotoxicity of T cells. In situations where relatively purified populations of T cells have been used, the T cells could bear IgM originally secreted by B cells. Both situations would require that a subpopulation of T cells possess IgM Fc receptors. In another series of experiments, we have in fact found a subpopulation of theta-bearing cells in mouse thymus which possess IgM Fc receptors (Lamon, E. W., B. Andersson, H. D. Whitten, M. M. Hurst, and V. Ghanta, unpublished observation and reference 21).

In summary, we have found that IgM, from mice which have undergone regression of primary MSV tumors, will induce cytotoxicity against the appropriate target cells by normal splenocytes and normal thymocytes. The thymocyte-induced cytotoxicity induced by IgM appeared to be more efficient than that produced by the same number of splenocytes. This was demonstrated by microcytotoxicity and ^{51}Cr-release assays.

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