ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY
IN THE MOLONEY SARCOMA VIRUS SYSTEM:
DIFFERENTIAL ACTIVITY OF IgG AND IgM
WITH DIFFERENT SUBPOPULATIONS OF LYMPHOCYTES*

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Adult murine hosts injected with Moloney sarcoma virus (MSV) develop
tumors at the site of inoculation which undergo spontaneous regression in a
high percentage of the animals (1). In vivo studies implicate thymus-dependent
cell-mediated immune mechanisms (2) as well as humoral immunity (3) in the
regression event. In vitro, specific antibodies (4–7) and lymphocytes (4, 8, 9–15)
from animals undergoing induction and regression of MSV tumors, have activity
against tumor target cells bearing the appropriate virally determined anti-
gen(s). Such animals develop humoral antibodies of both the M and G classes
which have specificity for the virally determined cell surface antigen(s) (5). IgM
as well as IgG, with specificity for the tumor target cells, can induce cytotoxicity
by normal lymphocytes and potentiate the cytotoxicity of immune lymphocytes
(16). Investigations into the subpopulations of cells involved as cytotoxic effector
cells against IgM-coated target cells led to the findings that splenic T and B cells
from normal or MSV regressor animals were induced to be cytotoxic or poten-
tiated in their cytotoxicity by immune IgM; nonphagocytic cells of the spleen
possessing neither surface Ig nor thymus-specific antigens were found not to be
active against IgM-coated target cells (17). Furthermore, normal thymocytes
were highly cytotoxic against the IgM-coated target cells (17, 18).

The purpose of the present investigation was to evaluate the differential
activity of IgG and IgM from MSV regressor animals in combination with
lymphocytes from spleen, lymph node, and thymus, and to determine whether
the antibody-dependent thymocyte-mediated cytotoxicity could be ascribed to a
particular subset of thymocytes.

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Abbreviations used in this paper: FCS, fetal calf serum; MEM, Eagle's minimal essential medium; MLV, Moloney leukemia virus; MSV, Moloney sarcoma virus.
Materials and Methods

Animals. Young adult BALB/c and CBA mice of both sexes, 6-12 wk of age, were used as sources of sera and lymphocytes.

Virus. MSV-B-64 was obtained from the Office of Progress Resources and Logistics, Viral Oncology, National Cancer Institute, National Institutes of Health, Bethesda, Md. This preparation has a titer of 6.2 log/FFU per ml on 3T3 cells. 0.1 ml of this preparation, when injected subcutaneously into adult mice, regularly produced tumors within 10-15 days after injection.

Inoculations. BALB/c mice were injected with 0.1 ml of the MSV suspension intramuscularly into the thigh. Local tumors developed as early as 5 days reaching a peak tumor size of 10-15 mm in diameter on day 15 and usually completely regressing by days 20-25. Regressor animals were bled at 30 days after the injection of virus, and the sera were pooled and heat inactivated at 56°C for 30 min. The data reported in this manuscript represent two serum pools collected from 10 animals each.

Antibody Fractionation. Serum was fractionated on Sephadex G-200 as previously described with a 90 x 2.5-cm column (16, 17, 19). The leading half of the first protein peak and the trailing half of the second peak were collected separately and reconcentrated to the original serum volume. With one of the serum pools (pool no. 1) the leading half of the 19S peak was refractionated on Sephadex G-200 and the leading half of this peak collected and reconcentrated. After reconcentration, IgM and IgG fractions were found to be free of cross-contamination by immunodiffusion and immunoelectrophoresis.

Medium. Eagle's minimal essential medium (MEM) with 10% heat-inactivated fetal calf serum (FCS) and 100 μg/ml of gentamycin was used.

Lymphoid Cells. Spleens, lymph nodes, and thymi from young adult CBA mice were dissected out under sterile conditions and prepared separately as follows. The organs were passed through fine nylon mesh into cold MEM without FCS, mixed with a Pasteur pipette, and passed over a fine nylon mesh filter. The cells were pelleted by centrifugation, and spleen cells were resuspended in Tris (hydroxymethyl-aminomethane)-buffered, 0.75% ammonium chloride, pH 7.2, and incubated for 10 min at 37°C to lyse the erythrocytes. This step was omitted with thymus and lymph node cells when erythrocyte contamination was minimal. The cells were washed once with MEM without FCS and resuspended in complete medium. 2-4 x 10^8 cells prepared as above in 5 ml of MEM were mixed with iron powder and incubated for 30 min at 37°C with agitation every 5-10 min. Iron and phagocytic cells were removed by passing the suspension through a series of five sterile tubes using a magnet to retain the iron in the discarded tube. The cells were then washed twice with MEM before use. The cells were counted in a hemacytometer, and appropriate dilutions were used in microcytotoxicity assays.

Thymocyte Fractionation. The approach taken in this series of experiments was to pharmacologically fractionate the thymocytes in vivo by injecting cortisone acetate, 6- to 8-wk-old CBA mice were injected with 2.5 mg of cortisone acetate. The thymus were harvested from such animals at 2 days after injection of cortisone when the thymus is reduced to approximately 5% of its original cell number and the remaining cortisone resistant cells have been shown to be immunologically competent (20). Also, thymi were harvested at 12 days after cortisone injection when the thymus has become repopulated to near normal size, and the cells have a lower immunologic reactivity than normal thymocytes (21). Processing of the lymphocytes was as described above. Thymocytes prepared in this way from control animals or after cortisone injection contain less than 1% B cells by direct anti-Ig immunofluorescence.

Target Cells. Ha2 is a cell culture line established from an MSV-induced tumor of a CBA mouse. These cells produce infectious virus, possess Moloney leukemia virus (MLV)-determined cell surface antigen(s), (4, 22) and have been shown to be sensitive to MSV immune lymphocytes in microcytotoxicity assays (4, 9-12), but not to lymphocyte immune to a nonpertinent antigen (9, 10, 23). These cells have also been shown to be sensitive to antibody-dependent cell-mediated cytotoxicity by using antibody with specificity for MLV-determined antigens and normal or MSV immune lymphocytes (16-18).

D-56 cells are sarcoma positive, leukemia negative cells derived from 3T3 NIH Swiss embryo fibroblast cultures. The cells possess the sarcoma genome, but do not produce any infectious virus nor do they possess any virally associated surface antigen that would distinguish them from normal 3T3 cells utilizing murine antisera (4). They are not sensitive to MSV immune lympho-
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cytes (4, 9-11). However, the same cells superinfected with MLV and thus possessing MLV-
determined antigen(s) are sensitive to MSV immune lymphocytes (4, 11). This cell line was
provided through the courtesy of Dr. R. H. Bassin, National Institutes of Health, Bethesda, Md.
Py3T3 cells are polyoma virus-transformed BALB/c 3T3 cells, kindly provided by Dr. Gene
Majors, Department of Microbiology, University of Chicago, Ill. These cells do not possess any
MLV-determined cell surface antigens, but are sensitive to immune lymphocytes from animals
that have been immunized against polyoma-transformed tumor cells (A. S. Walia and E. W.
Lamon, unpublished observation).

Microcytotoxicity Assays. A modification of the microcytotoxicity assay of Takasugi and Klein
(24) was used to determine antibody-dependent cell-mediated cytotoxicity (16-18). Briefly, 200 Ha2
cells in 5 μl vol of complete medium were seeded into each of the wells of microplates (3034 Falcon
Plastics, Div. of BioQuest, Oxnard, Calif.). Plates were incubated at 37°C in 5% CO2 for 3-4 h to
allow attachment of target cells. Serial dilutions of the antisera or their Sephadex G-200 fractions
were then added to the wells, six replicates per dilution in vol of 5 μl per well. The sera were
allowed to incubate with the target cells for 1 h, after which 10,000 lymphoid cells (or serial
dilutions as indicated in the Results section) were added to each of the wells. After incubation at
37°C in 5% CO2 for 20-24 h, the medium was removed and the plates were washed once with
balanced salt solution to remove dead cells. The plates were fixed and stained, and the target cells
remaining in each well or a representative portion thereof were counted. (Hereafter, a reference to
well indicates the number of target cells remaining in the well.)

Evaluation of Results

DETERMINATION OF PERCENT TARGET CELL REDUCTION BY LYMPHOID CELLS FROM SPLEEN, LYMPH
NODE, AND THYMUS. The log_{10} mean and standard deviation (SD) of the six replicate wells were
determined, and a Student's t test was performed to assess the significance of the differences
between test wells containing antibody and lymphocytes and the control wells containing only
lymphocytes. A significant difference was considered to exist at P values ≤ 0.05. Percent reduction
of the target cells in wells containing immune antibody and lymphocytes was calculated and
compared with wells containing lymphocytes only by using the geometric mean of the six replicate
wells for each serum dilution according to the following formula:

\[
\text{% reduction} = 100 - \frac{\text{Geometric mean of test wells}}{\text{Geometric mean of control wells}} \times 100
\]

The endpoint titer of an antiserum was considered to be the last dilution producing significant (P
≤ 0.05) target cell reduction compared to the control. As a further control, the antiserum and its
fractions were tested on control cells in combination with the same number and kind of lympho-
cytes as used on the Ha2 target cells.

CALCULATION OF Δ% REDUCTION. To compare the activity of thymocytes from the two exper-
imental groups of cortisone-treated animals, the change in percent reduction at each antibody
dilution was calculated according to the following formula:

\[
\Delta\% \text{ reduction} = \frac{100}{\text{surviving target cells with thymocytes from cortisone-treated animals}} - \frac{100}{\text{surviving target cells with control thymocytes}}
\]

To combine the data from more than one experiment, each sample containing thymocytes from
cortisone-treated animals was converted to a Δ% reduction value utilizing the mean percent
surviving target cells with control thymocytes and the same dilution of antibody for the denomina-
tor of the above formula. The arithmetic mean ± standard error of the Δ% reduction was then
calculated for each point of each experimental group with combined experiments. P values were
determined using a Student's t test to compare the percent surviving target cells from each
experimental group with the percent surviving target cells with control thymocytes tested with
the same fraction and dilution of antibody. The number of samples for each point in the experi-
mental groups was 12-18, and the number of samples in the controls was 24-30. Thus, the Δ% 
reduction indicates an increase or decrease in the ability of thymocytes from cortisone-treated
animals to be cytotoxic against the target cells in vitro, as compared to thymocytes from control
animals tested with the identical dilution and fraction of antibody. It should be pointed out that
the Δ% reduction calculated is antibody dependent. The number of surviving target cells incubated with thymocytes alone without antibody exhibited no significant differences between groups. From a single experiment the log₁₀ mean number of target cells remaining in wells with thymocytes only was 2.36 ± 0.10, 2.36 ± 0.26, and 2.35 ± 0.13 with control thymocytes, thymocytes 2 days after cortisone injection, and thymocytes 12 days after cortisone treatment, respectively.

Results

Both the IgM and IgG fractions of MSV regressor serum pool no. 1 were able to induce specific cytotoxicity by lymph node cells as indicated in Fig. 1. The end point titer for IgM against Ha2 cells was 1:80, and the end point titer for IgG against the Ha2 target cells was 1:40. This cytotoxicity was specific for the Ha2 target cells. No cytotoxicity was produced against Py3T3 cells with the same combinations of antibody and lymphocytes. Similarly no cytotoxicity was produced against D-56 cells with the same antibody and lymphocytes (data not shown). In addition to this specificity control shown in the figure, the antiserum or its Sephadex fractions produced no cytotoxicity against the Ha2 cells without the addition of lymphocytes or complement. Using similar controls, the antibody-dependent specificity was also confirmed with spleen and thymus effector cells.

Fig. 2 shows the ability of IgM and IgG from the same pool of serum to induce cytotoxicity by normal spleen cells against the Ha2 target cells. The end point titer for IgM was 1:160, and the end point titer for IgG was 1:20.

In contrast, thymocytes from control animals were exclusively cytotoxic in combination with IgM. No significant control thymocyte-mediated cytotoxicity was induced by IgG from this pool of regressor serum as indicated in Fig. 3. The end point titer of IgM in combination with thymocytes was 1:80.

To determine the relative efficiency of lymphoid cells from the different organs as effector cells against the antibody-coated target cells, the antibody fractions were placed on the target cells at a constant concentration (1:20) followed by graded numbers of lymphocytes from spleen, lymph node, or normal thymus. As indicated in Table I, when IgM was used as the sensitizing antibody, as few as 2,500 lymph node cells produced a significant target cell reduction. Thymus required 5,000 cells and spleen 10,000 to produce a significant target cell reduction against the IgM-coated targets. When IgG was used as the sensitizing antibody, both spleen and lymph node cells were about equally efficient as effector cells. Thymus cells, however, were not active against the IgG-coated tumor target cells.

The following experiments with thymocytes from cortisone-treated animals were performed with dilutions of antibody and a constant concentration of lymphocytes (10,000/well). The preceeding experiments were performed with serum pool no. 1, the following with serum pool no. 2.

Fig. 4 shows the differential activity of thymocytes from cortisone-treated animals compared to control thymocytes in the presence of unfraccionated MSV regressor antiserum. As indicated, the cortisone resistant thymocytes at 2 days after cortisone injection exhibited a very strong increase in cytotoxicity against Ha2 cells, whereas with the 12-day repopulated thymus, no significant change was evident. Also, against Ha2 target cells coated with the IgM fraction of the same antiserum, the 2-day cortisone resistant thymocytes
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**Figure 1.** IgM- and IgG-dependent cell-mediated cytotoxicity with lymph node effector cells; specificity of the reaction. The IgM and IgG were prepared from pool no. 1 MSV regressor sera described in the text. The solid horizontal line at 0 represents the activity of lymph node cells alone. The percent reduction at each antibody dilution was calculated according to the formula in the text. Points at or below the solid horizontal line indicate a lack of target cell reduction or stimulation of target cell growth, respectively. A second control target cell used in these experiments, D-56 cells, revealed essentially the same pattern as Py3T3 cells. The end point titer for IgM against Ha2 cells was 1:80. The end point titer for IgG against Ha2 cells was 1:40.

Produced a very strong increase in cytotoxicity, and there was a slight decrease in the cytotoxicity observed with the 12-day repopulated thymocytes (Fig. 5).

As indicated in Fig. 6, the 2-day cortisone resistant thymocytes were cytotoxic also against IgG-coated Ha2 target cells. This was not observed with control thymocytes (see Fig. 3 and Table I). Cells from the 12-day repopulated thymus exhibited little change from control thymocytes in combination with IgG, one antibody dilution producing a significant increase in target cell reduction and another antibody dilution producing a significant decrease in target cell reduction. (The latter represents a stimulation in target cell growth since IgG was not found to induce cytotoxicity with control thymocytes).

**Discussion**

We have thus presented an analysis of the effector cells involved in antibody-dependent cell-mediated cytotoxicity against tumor cells sensitized with IgG or IgM antibody from MSV regressor mice. With serial antibody dilutions and a constant concentration of effector cells (10,000/well), lymph node, spleen, and thymus were found to be active against IgM-treated target cells. Thymo-
Fig. 2. IgM- and IgG-dependent cell-mediated cytotoxicity with spleen cells as effector cells against Ha2 target cells. The percent reduction at each antibody dilution was calculated as in Fig. 1. The IgM and IgG were the same as those represented in Fig. 1. The end point titer for IgM was 1:160. The end point titer for IgG was 1:20.

Fig. 3. IgM- and IgG-dependent cell-mediated cytotoxicity against Ha2 cells with thymocytes as effector cells. The percent reduction was calculated as in Fig. 1. The IgM and IgG fractions are the same as those illustrated in Figs. 1 and 2. The IgG did not induce a significant cytotoxicity at any dilution. The end point titer for IgM was 1:80.
TABLE I

Lowest Concentrations of Lymphocytes Producing Significant Target Cell Reduction at Constant Antibody Dilutions

| Source of lymphocytes* | MSV-R antibody fraction |               | IgM | % red.$ | P|| | IgG |               | % red. | P   |
|------------------------|-------------------------|---------------|-----|---------|-----|-----|---------------|--------|------|
|                        | Dil.‡                   |               |     |         |     |     | Dil.          |        |      |
| Spleen                 | 10⁴                     |               | 17% | <0.05   |     | 10⁴ | 20%           | <0.005 |
| Lymph node             | 2.5 × 10⁵               |               | 27% | <0.005  |     | 10⁴ | 15%           | <0.0005|
| Thymus                 | 5 × 10²                 |               | 22% | <0.005  |     | None| None          | NS     | NS¶ |

Target cells used were Ha2 cells. The mean number of cells in control wells was 160 ± 19.5.
* Phagocyte-depleted lymphoid cells from control CBA animals tested at concentrations 2 × 10⁴,
  1.5 × 10⁴, 10⁴, 5 × 10³, and 2.5 × 10³ per well.
‡ Dilution: lowest number of lymphocytes per well giving significant target cell reduction in the
  presence of antibody.
§ % red.: percent reduction calculated by the formula in the text produced by the lowest dilution
  of lymphocytes giving significant (P < 0.05) reduction.
¶ P values obtained using a Student's t test compared to wells containing the same number of
  lymphocytes without antibody.
¶ NS, not significant.

Fig. 4. Change in antibody-dependent thymocyte-mediated cytotoxicity against Ha2 cells
after cortisone injection. The antibody used was unfractionated antiserum from MSV
regressor pool no. 2. The solid horizontal line at 0 represents the activity of control
thymocytes with the same antibody dilutions tested in an identical manner. Δ% reduction
was calculated according to the formula in the text and represents an increase or decrease of
cytotoxicity compared to control thymocytes tested with identical antiserum concentra-
tions. P values were derived using a Student's t test to compare each experimental group
with the control thymocytes at each antibody dilution. The vertical bars represent the
standard error of the Δ% reduction. ②, stands for days.
cytes were more cytotoxic against IgM-sensitized target cells than spleen and lymph node cells. Against IgG-coated target cells, lymphocytes from spleen and lymph node were cytotoxic but thymocytes were not. IgM induced a higher maximum cytotoxicity and end point titer than IgG with lymphocytes from all three organs. Using a constant antibody concentration, the number of lymphocytes required to produce cytotoxicity was evaluated. Lymph node cells and thymocytes were more efficient effector cells against IgM-coated target cells than spleen cells. Against IgG-coated target cells, spleen and lymph node cells were about equally efficient, and there was no observed cytotoxicity with normal thymocytes.

Utilizing cortisone as a means of in vivo fractionation, the major cytotoxicity observed with IgM and control thymocytes could be accounted for by the cortisone resistant subpopulation. In addition, although control thymocytes
were not induced to by cytotoxic by IgG, a significant cytotoxicity was produced against IgG-coated tumor cells when cortisone resistant thymocytes were used as the effector cells.

Previous work with splenic effector cells indicated that phagocytic cells do not contribute to this antibody-dependent cell-mediated cytotoxicity using the same target cells and the same assay system (16). Furthermore, cells possessing high concentrations of surface immunoglobulin (B cells), as well as cells possessing thymus-specific antigens (T cells), were found to be active against IgM-treated target cells. However, nonphagocytic spleen cells depleted of both B and T cells were not active (16). Since the thymocytes used in the present experiments contain less than 1% B cells and the suspensions were treated with iron powder and magnetism, it seems unlikely that the antibody-dependent cell-mediated cytotoxicity effector cells from the thymus are contaminating B cells or macrophages.

Antibody-dependent cell-mediated cytotoxicity has been described in a number of systems (25–29), including the MSV system (12, 16–18, 30–32). The antibody class responsible for this activity had been ascribed to IgG in some of these systems, and IgM was not found to be active. However, in two murine systems, both IgG and IgM have been found to induce cell-mediated cytotoxicity. We found that both IgG and IgM antibody from MSV regressor animals induced cytotoxicity by normal lymphocytes and potentiated the activity of immune lymphocytes against target cells bearing the appropriate virally determined surface antigen(s) (16). Also, Dennert and Lennox (33) reported that both IgM and IgG from mice immune to chicken erythrocytes (CRBC) induced CRBC lysis by lymphoid cells. More recently, Blair et al. (34) have reported arming activity exclusively in the IgM fraction in the mouse mammary tumor virus system.

One of the major concerns in the present series of experiments is the purity of the 19S antibody fraction. In previous reports, the 19S antibody from MSV regressor mice was shown to be IgM by size and by immunologic specificity (16). In the present report, the differential activities of the two antibody fractions in terms of their ability to induce cytotoxicity by lymphocytes from different organs provide additional evidence that the activities described are indeed due to IgM and IgG antibodies, respectively.

A number of previous studies (27, 28), including investigations reported by ourselves (6), had indicated that T cells were not required for antibody-dependent cell-mediated cytotoxicity. However, we have found that thymocytes and peripheral T cells can be involved in antibody-dependent cell-mediated cytotoxicity, particularly when IgM is used as the sensitizing antibody (17, 18). Furthermore, the present series of experiments demonstrate that thymocytes can be active against both IgM- and IgG-coated target cells.

The interaction of immune antibody and lymphocytes at the effector level may play a key role in the regression of MSV-induced tumors. Both humoral antibody and thymus-dependent cell-mediated immunity have been shown to be important in vivo for tumor resistance in this system. Antibody from regressor animals has been shown to protect previously unimmunized hosts against challenge with the appropriate syngeneic tumor cells (3). Also, it has been
demonstrated that the thymus is required for spontaneous regression of primary MSV tumors (2). The data described in the present report would seem to reconcile both the above groups of data (humoral and cellular) at the effector level. The thymus may be needed for the animals to produce an adequate antibody response against the virally determined antigen(s), and/or the thymus may provide cytotoxic effector cells. The latter could involve direct tumor cell cytotoxicity by immune lymphocytes or, as demonstrated in the present report, antibody-induced thymocyte-mediated cytotoxicity by a specific subpopulation of thymocytes. The structural bases for antibody-dependent thymocyte-mediated cytotoxicity would appear to be Fc receptors on T cells. This includes receptors for the Fc portion of IgG (35) and more recently demonstrated receptors for IgM Fc (36, 37). In addition to the implications in this particular tumor model, these data provide evidence that antibody secreted by B cells can direct functional events mediated by T cells, in this case cytotoxicity.

Summary

Antibody-dependent cell-mediated cytotoxicity in the Moloney sarcoma virus (MSV) system was evaluated in terms of the differential ability of IgG and IgM from MSV regressor animals to induce cytotoxicity by lymphocytes from lymph node, spleen, and thymus. The cell-mediated cytotoxicity induced by both IgM and IgG was specific for target cells possessing the appropriate virally determined cell surface antigen(s). IgM induced cytotoxicity by lymphocytes from all the organs tested. However, differences in magnitude and efficiency were revealed. Lymph node cells and thymocytes were most efficient against IgM-coated target cells. Against IgG-sensitized target cells, spleen and lymph node cells were about equally active, but thymocytes were inactive. Cortisone treatment of the donors of effector cells revealed that the cortisone resistant subpopulation of thymocytes, 2 days after cortisone injection, exhibited an increased cytotoxicity against target cells treated with unfractionated antiserum and its IgM fraction. This subpopulation of thymocytes was also cytotoxic against IgG-coated target cells. At 12 days after cortisone injection, the repopulated thymus showed little change in activity, compared to control thymus, against antibody-coated target cells.

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