Natural Antibodies Directed Against Murine Lymphosarcoma Cells [1, 2]

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SUMMARY—Natural antibodies reacting in a test of complement-dependent cytotoxicity with untreated murine lymphosarcoma cells of thymic origin were found in murine sera. Normal thymus cells were unaffected and unable to absorb the serum activity. The natural antibodies were IgM-like and stable at 56° C. They were not uniformly distributed in the studied strains, and high (C3H/He and C3Hf), intermediate (AKR and CBA/J), and low level strains (BALB/c, DBA/2, C57BL, and C57BL/6J) were found. Hybrids between a high (C3Hf) and a low level strain (C57BL) had the same response as the parental C3Hf mice. An inverse relationship was demonstrated between cytotoxicity of, and susceptibility to, serum of lymphoma cells in a given strain, which suggested that an immunologic modulation was at work. Embryonic cells absorbed the cytotoxic activity of the normal serum.—J Natl Cancer Inst 55: 945–949, 1975.

Serologic studies on mice demonstrated the presence of natural antibodies against autologous and allogeneic thymus cells (*1, 2*), endogenous type-C viruses (*3–5*), cryptic antigen(s) uncovered by neuraminidase (Nase) treatment on autologous, syngeneic, and allogeneic normal lymphoid cells (*6*), and recently also against tumor cells (*7*).

During the experiments on the characterization of tumor-associated antigens demonstrating the presence of embryonic-type structures on murine lymphoma cells (*8, 9*), we found, in murine sera, natural antibodies reacting with untreated lymphosarcoma cells and not affecting normal lymphoid cells. We investigated their characteristics and strain distribution and whether embryonic antigens interacted with them.

MATERIALS AND METHODS

Mice.—Inbred C57BL, C57BL/6J (B6), AKR, BALB/c, C3H/He, and C3Hf strains maintained for several years in this laboratory were used. The C3Hf strain was obtained in 1962 by cesarean section from a C3H/He female; foster nursing was by C57BL females. Sera were also harvested from CBA/J and DBA/2 mice provided by Dr. L. Chieco-Bianchi (Laboratory of Experimental Oncology, University of Padova, Padova, Italy).

Tumors.—Most tumors were thymic lymphosarcomas originally induced in this laboratory (*8*) by 7,12-dimethylbenz[*a*]anthracene (DMBA) in B6 (B6LyDMBA 1, 2), or by urethan (Ur) in C57BL (C57LyUr23, 24, 31, 32,) C3Hf (C3LyUr8, 11, 12), and BALB/c mice (BALyUrl), and kept in a subcutaneous transplant in syngeneic mice of the same sex as the tumor donors. In addition, 3 transplanted B6 lymphomas, i.e., the G virus-induced E♂G2 (*10*), the chemically induced EL 4 (*11*), and the radiation-induced ERLD (*12*), were also used.

Sera.—Three pools were obtained by collection of sera from the retro-orbital plexus of 2 groups of 80 adult C3Hf and C57BL males bearing a subcutaneous transplant of a syngeneic lymphoma (C3LyUr11, and 12, and C57LyUr24) and of 1 group of 140 normal 2- to 3-month-old C3Hf males. Pools of sera were also obtained from small groups of 3-month-old normal females of C3H/He, C3Hf, AKR, CBA/J, BALB/c, C57BL, B6, and DBA/2 strains. Antisera to embryo tissue produced by immunizing male C57BL mice with mitomycin C-blocked cells of 10- to 14-day C3Hf embryos [characteristics have been detailed in (*9, 13*)], were also used. Sera were stored at −30° C until tested and were not inactivated before use, unless specified.

A 2-ml aliquot of the C3Hf normal serum was chromatographed on a Sephadex G-200 gel filtration column (2.6×100 cm). The column, equilibrated and eluted with phosphate-buffered saline, was run at 12 ml/hour with an ascending flow; 6-ml fractions were collected. The fractions, corresponding to each of the three obtained peaks, were pooled and concentrated by ultrafiltration (Amicon PM-10; Amicon Corp., Lexington, Mass.) to the initial volume.

^{51}Cr cytotoxicity test for humoral response.—Cell suspensions were prepared from lymphomas or normal thymuses by the tissues being minced in Hanks' balanced salt solution (HBSS). They were repeatedly washed and adjusted at 20×10^6 living cells/ml in HBSS, and viability was determined by exclusion of trypan blue dye. The cells were then incubated with 200 μCi $Na_2{}^{51}CrO_4$/ml (Radiochemical Centre, Amersham, England) at 37° C for 45 minutes. The labeled cells were washed four times, adjusted to 1×10^6 cells/ml, and the mixture of 0.1 ml cells and 0.1 ml test serum at the proper dilution was incubated at 37° C for 30 minutes; three replicates were made for each sample. After incubation, 1 ml HBSS was added, the cells were sedimented by centrifugation, the supernatant was discarded, and 0.025 ml guinea pig complement (B.D. Merieux, Marseille, France), selected for absence of cytotoxicity on lymphoma cells and diluted 1:4, was added to two replicates, whereas the third one received 0.025 ml HBSS. The incubation was continued for 30 minutes longer. Then each tube was refilled with 2 ml HBSS, the cells were sedimented by centrifugation, and the radioactivity released in 1 ml of the supernatant was measured in a Packard Autogamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.)

We obtained the maximum amount of releasable radioactivity by incubating, at 37° C for 60 minutes, three samples, each with 1×10^5 labeled cells in 2 ml distilled water, and measuring the radioactivity of 1 ml supernatant after centrifugation. The percentage of specific ^{51}Cr release was calculated as follows:

$$\frac{\text{Experimental release} - \text{control release}}{\text{Maximum release} - \text{control release}} \times 100$$

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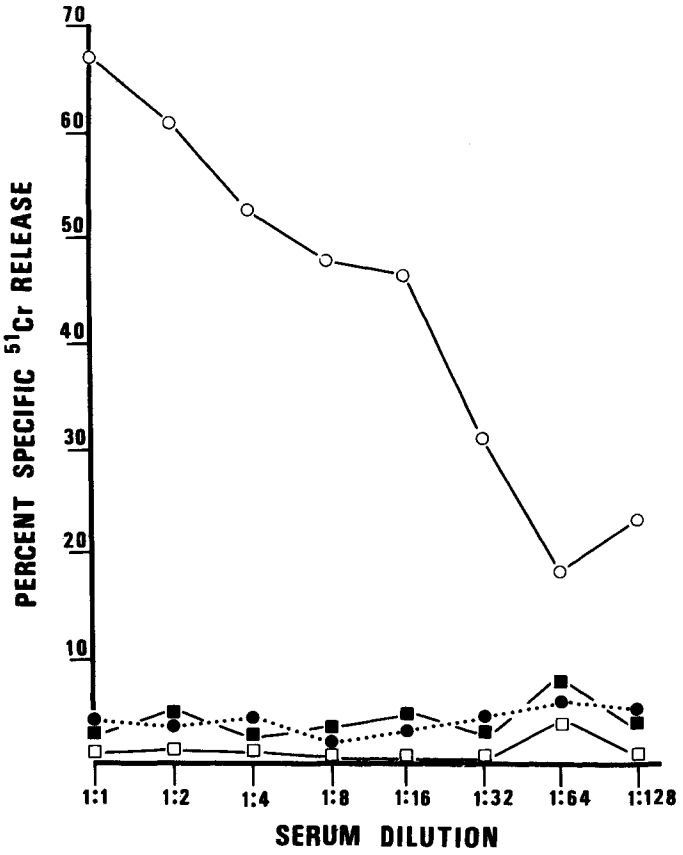
where the experimental release was the mean counts per minute (cpm) of the two replicates with complement, and the control was the radioactivity released after incubation with serum without complement. The control release for all tests had a mean value of 10.66±0.18% SE of the maximum. We controlled the complement cytotoxicity in each test by evaluating the ^{51}Cr release of three samples of cells incubated in HBSS and complement and of three samples containing HBSS only; the mean value for all tests was 3.72±0.49% SE of the maximum release.

RESULTS

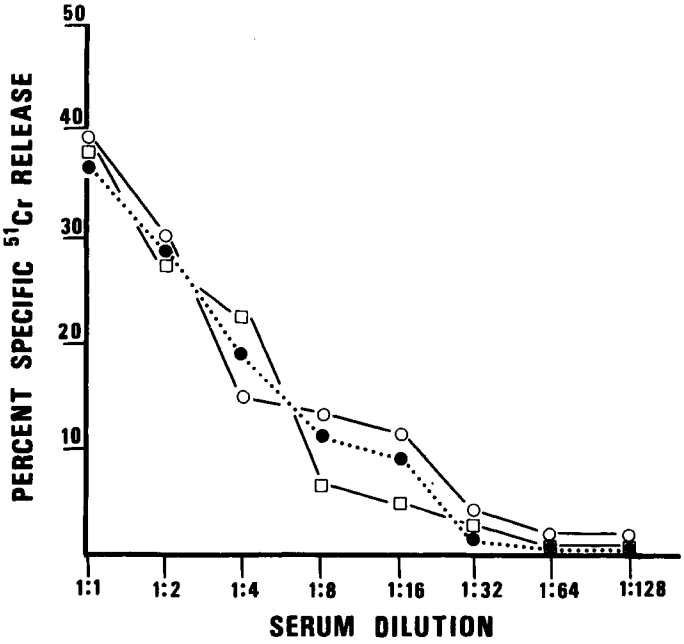
Cytotoxicity of Various Sera on Different Target Cells

The two pools of serum from C3Hf and C57BL male mice bearing a syngeneic lymphosarcoma were tested on C3Hf and C57BL lymphoma cells. As depicted in text-figure 1, the C3Hf serum exerted a high complement-dependent cytotoxic activity on C57LyUr24 target cells but not on syngeneic lymphoma cells, whereas the C57BL serum was ineffective on both types of target cells.

To verify whether normal mice had the same behavior, we performed tests to determine if serum from C3Hf normal males was capable of lysing the C57LyUr24 cells. In addition, the serum of C3H/He and C3Hf normal virgin females was tested on the same target cell to control a possible sex-linked effect or an influence of the original foster nursing of C3Hf mice on C57BL mothers. The three sera exerted the same level of complement-dependent cytotoxicity on the lymphoma cells (text-fig. 2).



TEXT-FIGURE 1.—Complement-dependent cytotoxicity: serum from tumor-bearing mice of C3Hf strain on C57LyUr24 (○——○) and on C3LyUr11 (●····●), of C57BL strain on C57LyUr24 (□——□) and on C3LyUr11 (■——■).



TEXT-FIGURE 2.—Complement-dependent cytotoxicity on the C57LyUr24 target cell of serum from C3Hf normal males (○——○), C3Hf (●····●), and C3H/He (□——□) normal females.

TABLE 1.—*Complement-dependent cytotoxicity of C3Hf normal undiluted serum on murine lymphomas induced in different ways*

Lymphoma target cells	Percent specific ^{51}Cr release±SE [a]
C57LyUr24	51.6±3.8[b]
C57LyUr23	41.2
C57LyUr31	29.3
C57LyUr32	41.3
B6LyDMBA1	35.6±5.8[c]
B6LyDMBA2	31.4
EL 4	54.2±4.1[d]
ERLD	54.3±6.5[e]
E♂G2	4.2–6.2
C3LyUr8	0.8–0
C3LyUr11	2.8±0.7[f]
C3LyUr12	0

[a] When not specified, 1 or 2 tests were done.
[b] Mean value of 8 tests.
[c] Mean value of 4 tests.
[d] Mean value of 9 tests.
[e] Mean value of 5 tests.
[f] Mean value of 3 tests.

The serum from C3Hf normal males was then tested at dilutions from 1:1 to 1:128 on various murine lymphomas induced in different ways and maintained in transplant in syngeneic C57BL, B6, or C3Hf mice. The results obtained with the undiluted serum, which gave the highest release in all instances, are reported in table 1. A strong, though variable, cytotoxic activity was observed on all chemically or radiation-induced C57BL and B6 lymphomas tested, whereas the E♂G2 leukemia of B6 strain and the chemically induced lymphomas of the C3Hf strain were unaffected at all serum dilutions.

Strain Dependence and Specificity of the Cytotoxicity of Normal Serum for Neoplastic Cells

Normal sera from C3Hf, AKR, CBA/J, BALB/c, DBA/2, and C57BL mice were tested at the same time on C57LyUr24 cells to compare their cytotoxic activity; in addition, 2 other lymphomas of C3Hf and BALB/c

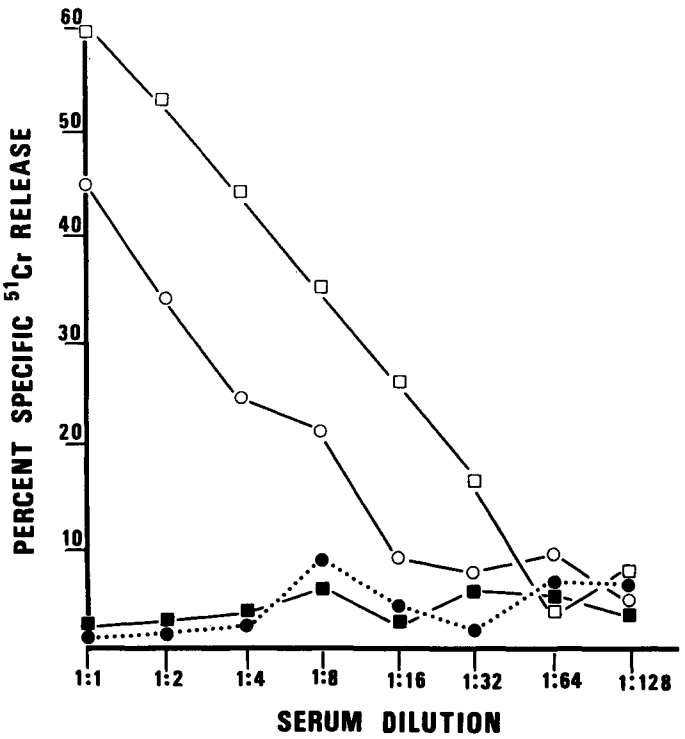
TABLE 2.—*Strain dependence of cytotoxic activity of normal mice sera and susceptibility of lymphoma cells*

Normal serum from [a]	Percent specific ^{51}Cr release from lymphoma:		
	C57LyUr24	BALyUr1	C3LyUr11
C3Hf	57.9	55.8	6.1
AKR	34.3	11.7	1.4
CBA/J	19.9	19.6	4.9
BALB/c	3.3	6.5	5.6
DBA/2	3.2	3.5	4.0
C57BL	1.6	6.5	1.9

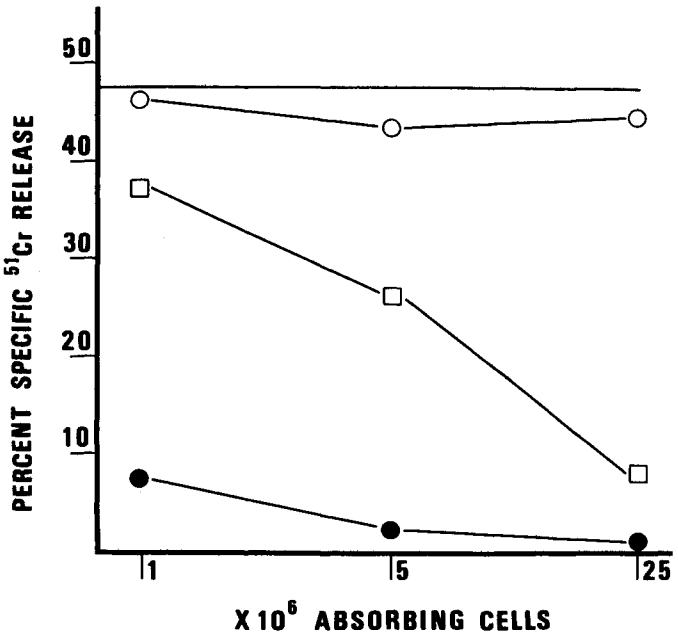
[a] Sera were tested from 1:1 to 1:128 dilution. Results represent the highest release, always obtained with undiluted serum.

strains were analyzed for their strain-dependent cell susceptibility to autologous and allogeneic sera. Various degrees of the natural response to C57BL lymphoma cells were found (table 2). Among the positive sera, the highest cytotoxicity was detected in C3Hf serum, whereas the CBA/J and AKR sera were cytotoxic at a lower level; BALB/c, DBA/2, and C57BL sera were negative.

In the three strains in which both serum and lymphoma cells were tested, an inverse relationship was detected between the cytotoxicity of serum and the susceptibility of lymphoma cells. In fact, we found that C3Hf, AKR, and CBA/J sera were cytotoxic for lymphoma cells of C57BL and BALB/c strains, the sera of which were negative on all target cells examined. Although the serum of C3Hf mice was positive on susceptible cells, their lymphoma cells were negative with the autologous serum. This lack of reaction was confirmed with the allogeneic AKR and CBA/J sera. The B6 strain had the same behavior as did the C57BL mice to serum and target cells (data not shown).



TEXT-FIGURE 3.—Complement-dependent cytotoxicity of C3Hf serum on C57BL (○——○) and BALB/c (□——□) lymphoma cells or on C57BL (●····●) and BALB/c (■——■) normal adult thymus cells.



TEXT-FIGURE 4.—Complement-dependent cytotoxicity on EL 4 cells of normal C3Hf serum unabsorbed (——) or absorbed with B6 normal thymus (○——○), C3LyUr11 (□——□), or EL 4 lymphoma cells (●——●).

To obtain information on the genetic control of the observed natural response against lymphoma cells, we tested the serum of hybrid mice obtained by crossing the highest (C3Hf) and the lowest (C57BL) responder strains on a susceptible target cell. The results demonstrated that the hybrids had the same response as the parental C3Hf mice (51.4 vs. 48.8% cytotoxicity with undiluted sera).

To control the specificity of the cytotoxicity on lymphoma cells, we tested C3Hf serum on C57BL and BALB/c lymphomas and on the corresponding normal thymus cells. C57LyUr24 and BaLyUr1 cells were sensitive to the cytotoxic factors, whereas C57BL and BALB/c thymus cells were unaffected (text-fig. 3).

To investigate the specificity further and to confirm the negativity of the C3Hf lymphosarcoma cells, we used a technique that would allow the C3Hf normal serum to be absorbed with various doses of normal B6 thymus, C3LyUr11, and EL 4 lymphoma cells, and then we tested it for residual cytotoxicity on EL 4 target cells. The results reported in text-figure 4 showed that thymus cells were unable to absorb the serum activity, whereas the C3Hf lymphoma cells absorbed the serum twenty times less efficiently than did the EL 4 cells. This suggested that a phenomenon, analogous to the cytotoxic negative–absorption positive seen in histocompatibility testing (*14*), was responsible for the negativity of the C3LyUr11 cells in the direct assay.

Cytotoxicity of Normal Serum Compared With Antiserum to Embryo Tissue

The C57BL antiserum to embryo tissue and the C3Hf normal serum were repeatedly tested on four lymphoma cells of different origin of C57BL and B6 strains. Both sera were found cytotoxic, although at different levels, on the three lymphomas induced by chemicals or by radiation, whereas the viral E♂G2 leukemia was insensitive to both (table 3). However, the E♂G2 leukemia was susceptible to lysis in a C'-dependent cytotoxicity

TABLE 3.—*Susceptibility of four lymphoma cells to C3Hf normal serum compared with C57BL antiserum to embryo tissue*

Lymphoma target cells	Percent specific ^{51}Cr release±SE with	
	C3Hf normal serum [a]	C57BL antiserum to embryo tissue [b]
C57LyUr24	51.6±3.8[c]	38.5±5.9[d]
EL 4	54.2±4.1[e]	61.3±11.7[f]
ERLD	54.3±6.5[g]	59.3−58.3
E♂G2	4.2−6.2	0−0

[a] Undiluted.
[b] Undiluted or diluted 1:2.
[c] Mean value of 8 tests.
[d] Mean value of 10 tests.
[e] Mean value of 9 tests.
[f] Mean value of 3 tests.
[g] Mean value of 5 tests.

test with an antihistocompatibility and an anti-G serum (unpublished data). The parallelism we found between the activity of the normal serum and antiserum to embryo tissue led us to assay by absorption the capability of embryonic structures to react with natural antibodies. The experiments showed that 10^7 embryonic cells absorbed 70% of the normal C3Hf serum activity for EL 4 target cells.

Characteristics of Cytotoxic Factors

No difference in cytotoxicity on C57LyUr24 cells was detected between the untreated C3Hf normal serum (44.5%) or after it was inactivated at 56° C for 30 minutes (45.5%).

The experiment with chromatographed C3Hf normal serum demonstrated that the cytotoxic activity of the unfractionated serum for C57LyUr24 cells (39.5%) was concentrated in the fractions from the first peak (37.9%), which was excluded from the gel and (on the column used) coincided with the IgM fraction of the serum, as confirmed by immunoelectrophoresis.

DISCUSSION

Studying normal sera from several mouse strains, we found a strain-dependent natural cytotoxicity for thymic lymphosarcoma cells. The cytotoxicity was complement dependent and did not affect untreated normal adult thymus cells.

Natural cytotoxic autoantibodies against thymus cells have been demonstrated in many strains of mice (*1, 2*). At present, they are not identifiable with the antibodies we found, since in our direct cytotoxicity studies, lymphoma but not thymus cells were affected; this was confirmed by absorption experiments. To avoid significant toxicity, we selected guinea pig serum as the source of complement as we did in other serologic studies on tumor-associated antigens, whereas Raff (*1*) and Martin and Martin (*2*) used hamster and rabbit sera, respectively. No lysis of thymus cells was observed by the last authors if rabbit serum was replaced with that of guinea pig as a source of complement. The difference in the complement used does not allow comparison of our results with those obtained on tumor cells by Martin and Martin (*7*).

An identity of the cytotoxic factors we found with the natural antibodies to endogenous type-C viruses detected in mice (*3–5*) is difficult to discuss because different techniques, such as radioimmunoassay and virus neutralization, were used. Hanna et al. (*5*) also tested the sera with the natural antivirus response in a cytotoxicity test, with negative results, but the target cells used, i.e., virus-induced lymphomas, may have conditioned the results. In our system, the only viral leukemia tested (the E♂G2) was insensitive to the natural serum activity. Therefore, it is difficult to compare the natural antivirus activity with the cytotoxicity we found on nonvirus-induced lymphomas, which may nevertheless express endogenous virus-induced antigens on the cell membrane.

The natural antibodies to lymphoma cells were present in four of the eight strains we studied; the C3Hf serum showed the highest level of cytotoxicity. The C3Hf lymphoma cells were consistently insensitive to normal C3Hf serum in the direct cytotoxicity test, which suggested a modulation phenomenon (*15*) that was not total because the tumor cells absorbed the activity of the serum, though less efficiently than did the B6 cells. Also supportive of the modulation hypothesis is the inverse relationship we detected within a given strain between the presence of natural antibodies to lymphoma cells and the susceptibility to lysis of the lymphoma cells.

On the other hand, the strain distribution of the response seems to indicate a genetic control of the expression of the cytotoxic factors, as suggested by the results obtained with sera from hybrids between the high and the low level strain; the character is dominant.

The hierarchy we observed in the cytotoxic activity of the serum of the various strains is analogous to that already seen on Nase-treated normal murine lymphoid cells (*6*). In addition we determined that the natural cytotoxins were IgM-like and stable at 56° C as were the natural antibodies found in human serum directed against receptors uncovered by Nase treatment in normal lymphoid cells (*16*). The hypothesis that the same structures present on normal cells (mostly in cryptic form and uncovered by Nase treatment) could be spontaneously exposed in neoplastic cells that have incompletely synthesized oligosaccharide chains of cell membrane glycoproteins is open to further investigation (*17, 18*). We have also shown that embryonic structures could be involved in our system.

We do not know the biologic significance of the observed natural reactivity. The widespread occurrence of natural antibodies in various mammalian species (*19–22*) suggests a control function of unknown physiologic phenomena. However, a trivial cross reaction with microbial antigens has not been ruled out.

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