ISOANTISERUM-AUGMENTED DEVELOPMENT OF LYMPHOCYTE-MEDIATED CYTOTOXICITY*

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Numerous studies have demonstrated that the humoral and cell-mediated immune response can be modulated by actively synthesized, as well as by passively transferred antibody (1–9).

The most frequently observed effect of passively administered antibody is the suppressed development of immunological responses by 7S antibodies (1, 2, 10–14). However, in certain circumstances both 7S and 19S antibodies have been shown to enhance the immune response (2, 3). While feedback control of the B-cell (bursa- or bone marrow-derived) response by passive antibody has been extensively studied, little information is available with regard to its effect on the development of T-cell responses (15). Studies have shown that delayed-type hypersensitivity (DTH)[1] appears less readily affected than the primary antibody response (1). In fact, Uhr and Möller (1) reported that development of DTH was facilitated by antigen-antibody complex. Liew and Parish (5) have shown that suppression of antibody formation was accompanied by a concomitant increase in DTH. These results, in addition to those reported by Mackaness and Lagrange (4), suggest that an inverse relationship often exists between DTH and the humoral immune response.

Here we report the effects of passively administered antibody on the in vivo development of lymphocyte-mediated cytotoxicity (LMC). Using an assay that has been demonstrated to measure T-cell cytotoxicity (16, 17), we show that administration of minute quantities of isoantiserum (IS) with low doses of allogeneic tumor cells facilitates development of cytotoxic T cells. We describe this as isoantiserum augmentation of T-cell development to avoid confusion with immune serum enhancement (8).

Materials and Methods

Cell lines. P-815-X2 mastocytoma cells were obtained from Dr. T. Brunner, Swiss Institute, for Experimental Cancer Research, Lusanne, Switzerland, and maintained by weekly transfer of 5×10^6 ascitic cells in DBA/2J mice (The Jackson Laboratory, Bar Harbor, Maine). EL-4 leukemia cells were obtained from Dr. E. A. Boyse, Memorial-Sloan Kettering Cancer Center, New York, and maintained in C57BL/6 mice.

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution, BSS-CS, BSS containing 10% calf serum; DTH, delayed-type hypersensitivity; H-IS, hyperimmune serum; I^o-IS, 10-day primary isoantiserum; IS, isoantiserum; LMC, lymphocyte-mediated cytotoxicity; NMS, normal mouse serum; PEC, peritoneal exudate cells; RPMI-CS, RPMI 1640 containing 10% CS.

Preparation of IS. EL-4 or P-815 cells were collected for immunization by aspirating the ascitic fluid from tumor-bearing mice. The cells were washed with balanced salt solution (BSS) by centrifugation at 200 *g* for 3 min. 1–3 × 10^7 EL-4 or P-815 cells were injected intraperitoneally (i.p.) into female BALB/c or male C57BL/6 mice, respectively. In most cases IS was obtained by bleeding mice 10 days after the first injection from the axillary area (I^o-IS). Antisera were pooled and heat inactivated by incubation for 45 min at 56°C. The cytotoxic titer of I^o-IS was 1/64 as determined by the method of Boyse et al. (18), with fresh rabbit serum as a source of complement and P-815 as the target cell. Hyperimmune serum (H-IS) was prepared by repeated weekly injections of 10^7 cells for a total of four doses. The cytotoxic titer of anti-P-815 H-IS was 1/512, while that for anti-EL-4 was 1/1,024.

Preparation of Peritoneal Exudate Cells (PEC) and Spleen Cells. Immune PEC for the cytotoxic assay were isolated 10–11 days after immunization, as described by Berke et al. (19). Briefly, the peritoneal cavity was washed out with 5 ml BSS containing 10% calf serum (BSS-CS) and 5 U heparin/ml. PEC were allowed to adsorb to the nylon wool (20) for 45 min at 37°C in a humidified CO_2 incubator. Unattached cells were eluted by permitting 30 ml of media to flow through the column. The eluted PEC were collected by centrifugation at 450 *g* for 5 min and suspended in RPMI-1640 containing 10% calf serum (RPMI-CS).

To prepare immune spleen cells, spleens were removed from immunized animals and teased apart in BSS. Cell debris was allowed to settle for 10 min at 0°C. Spleen cells were decanted, washed twice with BSS, and suspended in RPMI-CS.

Cytotoxic Assay. P-815 target cells were isolated from the ascitic fluid of tumor-bearing mice and labeled with ^{51}Cr as previously described (21). To measure in vitro LMC, 0.5 ml of suspensions containing 1 × 10^5 P-815 cells/ml and 5–50 × 10^5 immune lymphoid cells/ml were incubated in 35-mm plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Los Angeles, Calif.) on a rocking platform at 37°C in a humidified incubator (22). At several time points, ^{51}Cr released into the culture media was monitored, and the percent specific ^{51}Cr release was calculated as previously described: % specific ^{51}Cr release = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100 (23).

Sephadex G-200 Fractionation of Isoantiserum. A 2- to 3-ml sample of pooled anti-P-815 serum was applied to a Sephadex G-200 column (2.5 × 100 cm) and eluted by upward flow at a rate of 20 ml/h with 0.3 M NaCl in 0.1 M Tris-HCl, pH 7.4. The fractions eluting at the void volume containing IgM (Peak I) and the fractions containing IgG (Peak II) were pooled separately and concentrated at 4°C by pressure dialysis to the original serum volumes and kept frozen at −20°C until used.

Results

Effect of IS on the Induction of Lymphocyte-Mediated Cytotoxic LMC Activity. The effect of I^o-IS on the induction of lymphocyte cytotoxic activity was studied by immunizing C57BL/6 (*H-2b*) mice with allogeneic P-815 (*H-2d*) tumor cells coated with I^o-IS. I^o-IS was serially diluted in normal C57BL/6 serum to assure that each animal received an equivalent dose of serum protein. The diluted serum was mixed with P-815 cells, incubated at 37°C for 30 min, and an aliquot of 5 × 10^5 cells was injected i.p. per mouse. 10 days later LMC activity of PEC or splenic lymphoid cells was measured using ^{51}Cr-labeled P-815 as a target.

As shown in Fig. 1*a* PEC from mice immunized with mixtures of I^o-IS and tumor cells consistently showed significantly greater LMC activity than PEC from mice injected with P-815 alone or P-815 in BSS or P-815 in normal mouse serum (NMS). The observed I^o-IS effect on development of LMC activity occurred within a narrow range of I^o-IS dilution and decreased upon further dilution of I^o-IS. The I^o-IS augmentation of cytotoxicity was not restricted to PEC. Splenic lymphoid cells isolated from the same animals exhibited the same results (Fig. 1*b*).

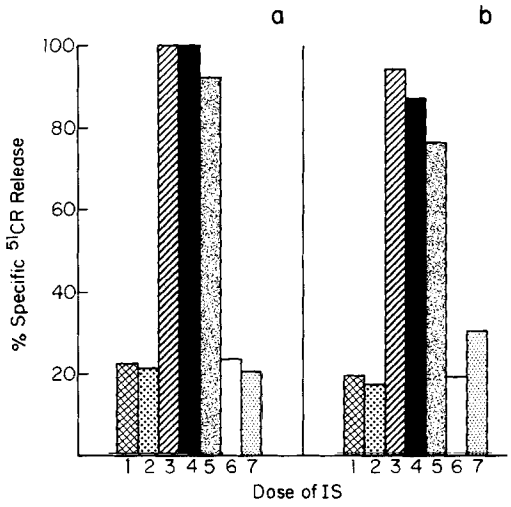

FIG. 1. IS augmentation of LMC activity. The primary IS was serially diluted in 1% NMS from the tube that contained 20 μl IS in 0.5 ml 1% syngeneic NMS-BSS. 5×10^6 mastocytoma cells were suspended in 0.5 ml of the 1% NMS-BSS containing various concentrations of I°-IS, incubated for 30 min at 37°C, and then aliquots of mixtures containing 5×10^5 cells were injected i.p. into C57BL/6 mice. Dose of IS: (1) BSS, (2) 1% NMS, (3) 2 μl, (4) 1.0 μl, (5) 0.5 μl, (6) 0.25 μl, and (7) 0.125 μl. (*a*) PEC were isolated and assayed for LMC activity at a lymphocyte to target cell ratio of 10:1 at the assay period of 2 h; (*b*) splenic lymphoid cells were assayed at a lymphocyte to target cell ratio of 50:1. The data are the mean of duplicate samples removed at 2 h and are representative of 10 experiments.

The IS-augmented induction of cytotoxicity was observed at doses of P-815 cells below that required for optimal stimulation of LMC activity. Therefore, it was possible that "augmentation" was simply explained on the basis of serum-stimulated growth of the P-815 in mice in the peritoneal cavity, since several other investigators have reported results to this effect (24).

If the I°-IS augmentation was merely serum-mediated increased proliferation of P-815, resulting in a greater antigenic dose in the IS-containing mixtures, the effect would be abrogated by irradiation. We found irradiated P-815 cells to be significantly less immunogenic than normal P-815. We, therefore, determined a minimal immunizing dose of 1,500 R irradiated P-815 to be in the range of 10^6 cells per mouse. Antibody-P-815 mixtures were incubated at 37°C for 30 min, then irradiated. As shown in Table I, irradiation significantly affects the immunogenicity of P-815 in that the cytotoxic activity of the PEC is low. The mice receiving 5 μl NMS show less cytotoxicity at all time points than mice receiving 5 μl IS, excluding the above described explanation for the IS augmentation of LMC.

Specificity of Isoantiserum-Augmented Induction of LMC Activity. Since several reports have described serum factors which nonspecifically induce LMC (25), the specificity of the I°-IS-augmented induction of LMC activity was investigated. The IS augmentation of LMC development was not unique to P-815. Another well-characterized allogeneic tumor system also showed similar results when IS-coated EL-4 tumor cells were used as the immunogen (Table II,

TABLE I
IS Augmentation of LMC Activity against Irradiated P-815 Cells

Incubation time	NMS	IS*
min	*% Specific ^{51}Cr release‡*	
180	6.6 ± 0.5	13.5 ± 0.1
270	13.0 ± 1.7	31.7 ± 1.7

* Irradiated P-815 cells were incubated with NMS or IS at 37°C for 30 min. The cell suspensions were subjected to 1,500 R X irradiation before injecting 10^6 cells per mouse with 5 μl of IS. This dose of irradiation did not allow growth of P-815 in DBA/2J.
‡ Data represent the mean of duplicate samples removed from the assay mixture at the time specified.

TABLE II
Specific IS Induction of Cytotoxic T-Cell Activity

Immunizing cell	Specificity of IS	Responder	Target cell	NMS	μl I°-IS/Mouse*			
					2.0	1.0	0.5	0.25
					% Specific ^{51}Cr release‡			
EL-4	Anti-EL-4	BALB/c (*H-2^d*)	EL-4	22.4 ± 2.8	9.5 ± 2.3	–	88.5 ± 5.1	–
P-815§	Anti-P-815	C57BL/6 (*H-2^b*)	P-815	17.2 ± 2.5	19.1 ± 1.1	21.9 ± 1.1	16.2 ± 0.4	–
–‖	Anti-P-815	C57BL/6	P-815	6.7 ± 0.4	7.0 ± 0.2	6.8 ± 0.8	5.4 ± 0.1	–
P-815 (*H-2^d*)	Anti-P-815	C57BL/6	P-815	8.6 ± 0.4	10.4 ± 0.1	23.1 ± 1.1	44.0 ± 2.0	6.1 ± 0.7
P-815	Anti-P-815	C57BL/6	EL-4	1.9 ± 0.1	2.0 ± 0.1	0.7 ± 0.8	1.5 ± 0.4	–
EL-4 (*H-2^b*)	Anti-P-815	BALB/c	EL-4	0.9 ± 0.3	4.5 ± 0.6	9.1 ± 2.1	3.5 ± 0.4	15.8 ± 0.2

* Dilutions of IS made in 1% NMS as described in Fig. 1.
‡ Data are from a 150 min time point at a lymphocyte to target cell ratio of 10:1, and represents the mean of duplicate samples from the same assay mixture.
§ P-815 cells incubated with IS above were washed twice in BSS, diluted to 5×10^6 cells/ml, and 5×10^5 P-815 cells were injected per mouse.
‖ IS incubated with 5×10^6 P-815 cells in 0.5 ml at 0°C for 30 min. The cells were pelleted, washed with BSS, and then incubated at 37°C for 30 min. The cells were pelleted. The supernates were diluted 1/5 and 0.5 ml was injected per mouse.

line 1). Anti-EL-4 antiserum increased the cytotoxic activity of PEC against EL-4 cells.

To further characterize the augmenting activity, we investigated whether antiserum-coated tumor cells functioned as well as antiserum tumor cell mixtures in augmenting induction of LMC activity. When P-815 cells were separated from IS after 37°C incubation, PEC from mice receiving the cell pellets (line 2) and those from mice receiving culture supernates (line 3) failed to induce detectable levels of LMC 10 days after immunization. Thus, IS augmentation was reproducibly observed only when whole mixtures of cells and IS are injected together.

PEC from mice immunized with I°-IS-coated P-815 cells showed cytotoxicity specific for the alloantigens of P-815 (*H-2d*) (line 4), because PEC from mice showing IS-augmented cytotoxic activity showed no detectable cytotoxicity toward EL-4 (*H-2b*) (line 5). Anti-P-815 serum administered with EL-4 cells failed to augment development of LMC against P-815 (line 6). Furthermore, when anti-P-815 IS was adsorbed with P-815 cells (10^8 cells/ml IS) to remove in vitro LMC-blocking activity (23), augmenting activity of IS was also removed from the serum (Table III). Anti-P-815 IS absorbed with EL-4 leukemia cells as a

TABLE III
Removal of IS-Augmenting Activity after Absorbing Serum with P-815

Absorbing cell	Exp. no.	NMS	μl I°-IS serum per mouse*			
			2.0	1.0	0.5	0.25
			% Specific ^{51}Cr release			
–	1	9.1 ± 0.4	9.0 ± 0.0	13.2 ± 0.2	15.9 ± 0.0	29.6 ± 2.0
	2	24.6 ± 0.6	19.3 ± 0.3	57.0 ± 0.8	38.3 ± 0.4	80.5 ± 1.4
EL-4‡	1	–	58.7 ± 3.0	37.2 ± 0.1	29.1 ± 1.4	21.6 ± 0.6
	2	–	46.4 ± 1.8	64.8 ± 1.3	46.7 ± 2.1	73.5 ± 2.8
P-815‡	1	–	19.0 ± 0.2	18.7 ± 0.1	7.3 ± 0.1	16.2 ± 0.3
	2	–	22.7 ± 0.1	16.6 ± 0.3	10.5 ± 0.2	2.1 ± 0.1

* IS diluted in 1% NMS, as in Fig. 1.

‡ 1.0 ml of serum was adsorbed three times with 1×10^8 EL-4/ml serum. The serum was divided into 0.5-ml aliquots. 0.5 ml was further adsorbed three times with 1×10^8 P-815/ml of serum. Data represent the mean of duplicate samples removed from assay mixture at 180 min at a lymphocyte to target cell ratio of 10–15:1.

control retained the capacity to increase cytotoxic T-cell activity. We found in these studies that the nonspecific absorption with EL-4 actually increased the augmenting activity of the I°-IS. Possibly, the absorption removes autoantibodies that counteract the augmenting activity of the serum. These results show that the serum factor responsible for augmenting cytotoxic T-cell development possessed immunological specificity. It was, therefore, important to show that Ig was the serum component causing the effect.

G-200 Sephadex Fractionation of I°-IS and H-IS. Primary and hyperimmune serum were fractionated on G-200 Sephadex. The pooled peak fraction (Peak I) eluting at the void volume contained IgM, as identified by immunoelectrophoresis with goat antimouse-Ig, and the second peak which was retained in the column (Peak II) contained IgG.

As shown in Table IV, Peak I from I°-IS showed in vivo augmentation of LMC activity; PEC from mice receiving P-815 coated with Peak I of I°-IS were significantly more cytotoxic (41% at a 0.5 μl/mouse dilution vs. 7% in the control) than PEC from mice receiving Peak I of H-IS (28% at a 2 μl/mouse dilution).

The pooled G-200 fractions from I°-IS and H-IS were also assayed for their ability to block in vitro LMC (Table V). Primary serum has little detectable blocking effect on LMC against P-815 (line 1). The hyperimmune serum, however, shows significant blocking activity in peak II (line 3) (19% kill vs. 59% found with NMS Peak II) as previously described (23, 26).

Since IgM production precedes that of IgG, we investigated whether or not augmenting activity coincided with the development of blocking activity. C57BL/6 mice were immunized with 10^7 DBA mastocytoma cells and bled 10 days later, before a booster immunization. The weekly bleeding and immunization were repeated four times. The serum from each bleeding was monitored for its capacity to augment the in vivo development of cytotoxic T cells by injecting it with P-815 cells (Table VI) and for its capacity to block in vitro LMC (Fig. 2).

TABLE IV
Augmenting activity of G-200 Fractionated Primary and Hyperimmune Serum

	NMS	μl of fraction/mouse*			
		2.0	1.0	0.5	0.25
		% Specific ^{51}Cr release‡			
I^0-IS PK I	6.9 ± 0.3	4.9 ± 1.1	20.3 ± 0.4	41.1 ± 1.6	1.7 ± 0.5
I^0-IS PK II	5.2 ± 1.2	14.9 ± 0.8	14.6 ± 1.2	21.9 ± 1.1	14.8 ± 0.1
H-IS PK I	6.1 ± 0.9	28.8 ± 2.6	–	7.1 ± 1.3	7.0 ± 0.1
H-IS PK II	6.1 ± 0.9	6.5 ± 1.0	–	3.1 ± 0.2	5.6 ± 0.0

* IS diluted in 1% NMS, as in Fig. 1.
‡ Data represent one of three experiments, the mean of duplicate samples removed from the assay mixture at an assay time of 240 min at a lymphocyte:target cell ratio of 5:1.

TABLE V
Blocking of LMC by G-200 Fractionated 10-Day Anti-P-815 and H-IS

Serum fraction	NMS*	I^0-IS	H-IS
		% ^{51}Cr release‡	
Whole mouse serum	53.3 ± 1.5	60.2 ± 0.6	12.2 ± 0.8
PK I	58.1 ± 0.3	59.2 ± 1.8	58.0 ± 0.1
PK II	59.4 ± 1.3	50.2 ± 0.4	18.6 ± 0.6

* Antiserum added to final concentration of 5% in the assay mixture.
‡ Results represent the mean of duplicate samples removed at 150 min at a lymphocyte to target cell ratio of 10:1.

TABLE VI
Chronological Development of Immune Serum Augmenting Activity

No. of immunizations*	Days of bleeding‡	NMS	μl Serum per mouse		
			1	5	10
			% Specific ^{51}Cr release§		
1	10	17.8 ± 1.8	23.0 ± 0.8	29.3 ± 4.2	44.7 ± 3.7
2	17	1.9 ± 0.2	5.2 ± 0.1	65.3 ± 2.3	3.8 ± 0.6
3	24	17.7 ± 0.1	39.6 ± 0.7	37.1 ± 1.2	–
4	31	0.4 ± 0	28.7 ± 0.5	2.3 ± 0.4	–

* Mice received 1×10^7 cells per booster.
‡ Days after first immunization (Fig. 2).
§ Data represent the mean of duplicate samples removed at 150 min at lymphocyte to target cell ratios of 13:1.

A significant level of augmenting activity was present in the serum after the first immunization (Table VI, line 1). As the number of immunizations increased, it was more difficult to consistently demonstrate the serum-augmenting activity as evidenced by the data in Table VI (lines 2–4). The inconsistencies in augmentation of LMC activity appear when serum-blocking activity became more pronounced (Fig. 2 and Table VI, lines 3 and 4).

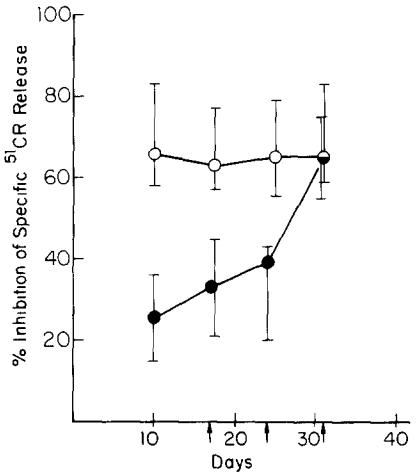

FIG. 2. Chronological appearance of in vitro LMC-blocking activity. LMC-blocking activity at the assay period of 2 h was monitored on the day of bleeding before a booster injection of 10^7 P-815 cells, as indicated by arrows, (●); control experiments with hyperimmune sera were performed each time, (○). The data represent the mean of three experiments.

Discussion

Using an assay system which measures "pure" T-cell immune responses (16, 17), we show that passive administration of IS with allogeneic tumor cells modulates the induction of LMC activity. We present evidence that the factor in IS is likely to be specific antibody induced by immunization: (*a*) both in vivo augmenting and in vitro blocking activities are present in IS; (*b*) both activities are removed by absorption with specific cells; and (*c*) demonstration of the augmentation effect requires injection of tumor cells plus IS together. The augmentation effects were best manifested when mice were immunized with low doses of tumor cells.

The IS-augmented induction of LMC activity is similar to the enhanced development of PFC when mice received antiserum and SRBC together (2, 3). These studies demonstrated that 19S antibodies may stimulate PFC formation, whereas 7S antibodies usually suppressed. Henry and Jerne (2) also emphasized that stimulation of PFC by immune sera required low doses of antigen. In agreement with these observations, our results showed that the IS effects on induction of LMC activity was best manifested when the dose of immunizing tumor cells was low. Our experimental results suggest that the augmenting factor in immune serum was 19S antibody, because the activity appears within 10 days after a primary injection and migrates with the 19S fraction in G-200 chromatography. Development of in vivo augmenting activity, after immunization, occurs before significant levels of in vitro LMC-blocking activity occurs in the serum (Table V and Fig. 2).

It is known that administration of serum from tumor-bearing animals can result in enhanced tumor growth (8). In these cases, 7S antibodies appear to be responsible for the enhancement (10–12). On the other hand, evidence is also accumulating that indicates that passively administered immune sera may suppress rather than enhance tumor growth (27–29). The latter studies failed to

show whether or not 7S antibodies were involved in suppression of the tumor growth.

The presence of an antigen-antibody complex in the sera of tumor-bearing mice has been known (30). A paradox has evolved, however, with regard to the role of immune complexes in regulating expression of immunological functions. Several investigators have suggested that immune complexes suppress both the humoral and cellular immune response (31, 32), while others have reported stimulation (33). These contrasting results are reconcilable if one considers manifestations of the antigen vs. antibody composition of the immune complex (34, 35) or the physiological difference between 7S complex and 19S complex. It appears possible that the development of either a humoral or cellular immune response is delicately balanced between the level of 7S vs. 19S antibody production and the consequent formation of antigen-antibody complex.

The exact role of passively administered antibody in augmenting T-cell responses is presently not understood. Our experimental evidence is unique in revealing that antigen-antibody complex has a profound effect on development of LMC activity. It does appear that formation of the antigen-antibody complex is intimately involved in influencing expression of specific T-cell activity. We have found, as have others, that antibody and antigen must combine in optimal proportions to achieve the observed effects on LMC activities (Fig. 1), although the influence of IgG-immune complex vs. IgM-immune complex influence on development of immune responses has yet to be delineated. Our data indicates that IgM-immune complex augments development of LMC while the IgG-immune complex works in an opposing fashion.

Playfair (15) has postulated that antigen binding, augmentation of antibody production, and suppression of antibody production can be mediated by passively acquired Ig on the surface of T cells, suggesting that Ig can attach to T cells with or without being complexed with antigen. In fact, Hudson and Sprent (36) have recently shown that IgM antibody is specifically adsorbed to H-2-activated mouse T lymphocytes.

The described observations tempt one toward a simple hypothesis. Inbred "normal" mice are now known to contain significant amounts of natural antibody (37); therefore, they may contain low levels of alloactivated T cells. The passive administration of IgM with antigen negates the animal's requirement to produce an endogenous supply. Macrophages recognize the IgM-antigen complex, and through their digestion produce processed IgM-antigen complexes, which adsorb to the omnipresent, activated T cells initiating clonal expansion of the specific cytotoxic T cell. This proposal is currently being investigated in this laboratory.

Summary

Passive administration of anti-P-815 isoantiserum (IS) with P-815 mastocytoma was shown to augment the development of T-lymphocyte-mediated cytotoxicity (LMC). The LMC activity augmented by IS was specific to immunizing tumor cells, but required the simultaneous administration of P-815 tumor cells and anti-P-815 serum, suggesting that the antigen-antibody complex is involved in the development of specific cytotoxic T cells.

The serum component responsible for augmented development of LMC activity appeared to be IgM in that the augmenting activity fractionated in the void volume of a G-200 Sephadex column and appears very early after immunization. Our experimental results suggest the development of specific T-cell cytotoxicity can be directly regulated by specific IgM antibodies.

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