

THYMUS-DERIVED LYMPHOCYTES PRODUCE AN
IMMUNOLOGICALLY SPECIFIC MACROPHAGE-
ARMING FACTOR*

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Peritoneal macrophages from nonimmunized mice become specifically cytotoxic after incubation with either immune lymphoid cells (1) or cell-free supernatants from cultures consisting of sensitized lymphoid cells and specific target cells (2). Similar supernatants also rendered glass-adherent bone marrow cells specifically cytotoxic (8). Experiments described in this report show that these supernatants contain a specific macrophage-arming factor (SMAF) whose molecular weight indicates that it is too small to be an intact immunoglobulin, yet at the same time has a specific recognition site for the target cell. In these experiments, immune reactions are probably directed against normal transplantation antigens since the mice were immunized with allogeneic target cells. However, parallel studies in purely syngeneic combinations where the antigenic disparity consists only of tumor-specific antigens reveal that the properties of SMAF produced in the syngeneic system are very similar to those of SMAF produced in the allogeneic.

The design of these experiments is intended to demonstrate that SMAF production is dependent on the presence of thymus-derived lymphoid cells (T cells).

Materials and Methods

Mice.—Pure line CBA, DBA/2, and C57/BL mice 8–10 wk of age were used.

Tumors.—The SL2 and TLX9 lymphomas, syngeneic for DBA/2 and C57/BL mice, respectively, grow as peritoneal ascites tumors. Their origin has been reported elsewhere (3). Cells used for immunization purposes or growth in cultures were aspirated from the peritoneal cavity 7 days after implantation and washed three times in Fischer's medium.

Cell Cultures.—(a) Lymphoma cells were seeded in growth medium, Fischer's medium containing 10–15% heat-inactivated fetal bovine serum, at a concentration of 10^5 cells/ml. The generation time of SL2 is 13–16 hr, that of TLX9 is currently 24 hr. (b) Peritoneal macrophage monolayers were prepared as described fully elsewhere (1). Approximately 1.6×10^6 macrophages in growth medium were seeded into 3-cm Sterilin dishes. (c) Spleens were removed from immune or nonimmune CBA mice and cell suspensions were prepared by the method of Denham et al. (4).

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Immunization.—CBA mice were injected intraperitoneally with 5×10^6 dividing SL2 or TLX9 cells. Mice thymectomized at 10 wk of age, irradiated, and reconstituted with bone marrow cells (5) were kindly supplied by Dr. A. J. S. Davies of this Institute. These T cell-deprived mice could not reject a challenge of dividing lymphoma cells and were immunized with two injections of 5×10^6 irradiated (5000 R) lymphoma cells 10 days apart. Sham-operated CBA mice, that is mice which received a "sham" thymectomy operation and were irradiated and reconstituted, were similarly treated. Spleens were removed from immunized mice 14 days after the final immunization.

Anti- θ Serum.—The method used was a modification of that reported by Reif and Allen (6). AKR mice received four intraperitoneal injections of 2×10^7 C3H thymocytes separated by 3-wk intervals, the first being given in conjunction with *Hemophilus pertussis* vaccine (10^9 organisms per mouse) as an adjuvant. Blood was collected from the axilla 14 days after the final injection, pooled, and the serum separated. This antiserum was cytotoxic in the presence of 1/10 complement (New Zealand White rabbit serum, absorbed with packed SL2 cells) to mouse thymocytes and to 30% of spleen cells. Neither normal nor immune peritoneal macrophages were affected by this treatment.

Production of SMAF by Immune Spleen Cells.—Cultures of spleen cells and irradiated lymphoma cells were prepared (10^7 spleen cells: 10^5 lymphoma cells per milliliter) and incubated for 24 hr at 37°C (2). At this time the culture fluids were removed, centrifuged, and tested for their ability to render nonimmune CBA macrophages cytotoxic to target cells in an immunologically specific manner (see below).

Fractionation of SMAF.—Culture supernatants were concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) using a PM.10 membrane, and 1 ml of concentrate was placed on a Bio-Gel A 0.5 m agarose gel column. The excluded material (F1), mol wt $>300,000$, was concentrated and dialyzed, while the fractions eluted after this were pooled and concentrated to 1.5 ml. 1 ml was then placed on a Bio-Gel P-150 polyacrylamide gel column. The eluate from this column was divided into three main fractions corresponding to molecular weight sizes of approximately 100–300,000 (F2), 50–100,000 (F3), and 20–50,000 (F4) daltons, respectively. The fractions were dialyzed and reconstituted with Fischer's medium free of serum.

Macrophage Cytotoxicity Test.—This test is based on the capacity of macrophage monolayers treated with SMAF to prevent growth of target cells. The reaction involves initially a cytostatic mechanism whereby target cells fail to divide, and this is followed by their death and disintegration. Cell-to-cell contact is required but phagocytosis of intact cells is not seen and occurs only when cells are disintegrating. The technique and method of assessing growth inhibition is described fully elsewhere (1).

RESULTS

To demonstrate that SMAF possesses a specific recognition site for the target cell as well as a cytophilic moiety for macrophages (2), SL2 or TLX9 cells were incubated with SMAF produced by stimulation of spleens from specifically immunized CBA mice, and also with SMAF directed against the unrelated target cells. Table I shows that target cells treated with SMAF directed against them failed to grow in the presence of macrophages, whereas target cells incubated with unrelated SMAF (i.e. SL2 cells treated with SMAF directed against TLX9 cells) grew normally. SMAF did not affect growth of the specific target cells in the absence of macrophages. SMAF could be totally absorbed out by specific target cells but not by unrelated cells. The cytophilic moiety was also able to bind to rat macrophages in a nonspecific manner.

SMAF directed against SL2 cells was fractionated, as described in Materials and Methods, and four main fractions (F1-F4) were collected. Serial fivefold dilutions of each fraction were prepared in growth medium and SL2 and TLX9 cells were incubated with each dilution for 1 hr at 37°C. The cells were then

TABLE I
Growth Inhibition of SL2 or TLX9 Cells after Incubation with Supernatants from Immune Spleen Cell Cultures with or without Target Cells

Culture supernatant obtained from	Percentage growth inhibition of lymphoma cells 48 hr after treatment with the various supernatants and seeding onto macrophage monolayers*	
	SL2	TLX9
	%	%
Spleen cells from CBA mice immunized with SL2 cells and then incubated with		
Irradiated SL2 cells	88 ± 5	4
Irradiated TLX9 cells	8	6
No cells added	2	3
Spleen cells from CBA mice immunized with TLX9 cells and then incubated with		
Irradiated TLX9 cells	5	86 ± 8
Irradiated SL2 cells	5	2
No cells added	2	4

* The lymphoma target cells were incubated at 10^6 cells/ml of supernatant for 1 hr at 37°C, washed 3 ×, and resuspended in growth medium. 3 ml containing 10^5 cells were added to dishes with or without macrophages. Control cultures consisted of treated and untreated target cells grown in the absence of macrophages, or untreated cells added to normal macrophage monolayers. In all of these, SL2 cells grew to $1.5 \times 10^6 \pm 10\%$; TLX9 grew to $0.5 \times 10^6 \pm 10\%$ over the 48 hr period.

TABLE II
The Effect of T Cell Deprivation by Anti-θ Serum and Adult Thymectomy and Whole Body Irradiation on the Ability of Immune Spleen Cells to Produce SMAF

Macrophages incubated with supernatants produced by immune spleens (+ SL2 cells) from	No. of SL2 cells ($\times 10^6$) at 48 hr
Control immunized mice	0.2 ± 0.2 (87%)
“ “ “ + treated with anti-θ serum*	1.4 ± 0.2 (1%)
Control unimmunized mice	1.5 ± 0.1
“ “ “ + treated with anti-θ serum	1.4 ± 0.1
T cell-deprived mice	1.3 ± 0.1 (14%)§
Sham-operated mice‡	0.5 ± 0.1 (67%)

* Spleen cells treated with anti-θ serum before the addition of SL2 cells.

‡ Whole body irradiated and reconstituted with bone marrow but not thymectomized.

§ Growth inhibition after seeding 10^5 cells per monolayer.

washed thoroughly and placed on macrophage monolayers to see whether they grew or not. The data from numerous experiments indicated that SMAF contains two major peaks of activity, the one containing material of molecular weight greater than 300,000 (F1), the other between 50 and 100,000 (F3). TLX9 cells grew normally in the presence or absence of SL2-directed SMAF.

When the individual tubes comprising the pooled F3 fraction were titrated, the SMAF activity was found to be associated with a molecular weight size of 50–60,000.

The involvement of T cells in the production of SMAF was shown in two ways (see Table II): firstly, immune spleen cells exposed to anti- θ serum and complement did not produce SMAF on exposure to specific target cells, while spleen cells not exposed to anti- θ gave a positive response to stimulation by target cells. Secondly, SMAF could not be obtained from spleens of CBA mice which had been thymectomized, irradiated, reconstituted with bone marrow, and immunized with two injections of irradiated SL2 cells (Table II). However, spleen cells from control sham-operated mice produced SMAF in response to stimulation with specific target cells. T cell-deprived mice after immunization produced cytotoxic antiserum but no cytotoxic lymphocytes, macrophages, or arming spleen cells, while sham-operated mice produced not only cytotoxic serum but also cytotoxic lymphocytes, macrophages, and arming spleen cells (data to be published).

DISCUSSION

A plethora of biologically active substances are released when lymphocytes from immunized donors are stimulated with specific antigens. These so-called lymphokines have been considered to be a product of the T cells derived from the immunized animals (7). The present study would support this contention, that under conditions where one would expect lymphokines to be produced, a population of T cells is required for the formation of SMAF. On fractionation SMAF appears to consist of two major components, the one being $>300,000$ daltons, the other 50–60,000 daltons. Both have been shown to bind (*a*) in a specific manner to the target cells used to sensitize the host and (*b*) in a totally nonspecific manner to mouse and rat peritoneal macrophages. Studies are now in progress to determine if the antigen recognition site of SMAF is similar to that of immunoglobulins and whether SMAF has determinants, perhaps those of a light chain, which cross-react with known immunoglobulins. Previously (8) we had found that a SMAF-like material could be obtained when cells from the thymus of immunized mice were cultured with specific target cells. These thymus cells were not cytotoxic and this suggests that the capacity to make SMAF is not necessarily associated with a population of T cells which are cytotoxic, although in spleen and lymph nodes there is another class of T cells which is directly cytotoxic towards specific target cells. A similar situation has been encountered in mice immunized with syngeneic lymphoma cells in which the lymphoid cells which were not directly cytotoxic *in vitro* (9) produced SMAF (2). The relationship of SMAF to the immunologically specific macrophage migration factor described by Amos and Lachmann (10) and to some other immunologically specific T cell products discussed by Mitchison et al. (11) is not yet known.

SUMMARY

Spleen cells from mice immunized with an allogeneic tumor when cultured with the specific tumor cells release into the supernatant a specific macrophage-arming factor(s) (SMAF) which binds nonspecifically to macrophages from both mice and rats and renders these cytotoxic to the specific tumor cells. SMAF also binds in an immunologically specific way to the target cells. SMAF-treated target cells grow normally in the absence of macrophages but are killed in the presence of normal macrophages. Thymus-derived cells are necessary for the production of SMAF since (a) after treatment with anti- θ serum immune spleen cells fail to release SMAF; (b) spleen cells from immunized T cell-deprived mice (thymectomized as adults followed by whole body irradiation and restored with bone marrow) fail to produce SMAF on stimulation with the specific target cells. While SMAF has the properties of a cytophilic antibody, it does not belong to one of the established classes of immunoglobulin since high activity is found after column separation in a fraction having a molecular weight between 50,000–60,000 daltons.

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