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Suppression of antitumor immunity by macrophages in spleens of mice bearing a large MOPC-315 tumor

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Summary. *We had shown previously that progression of MOPC-315 plasmacytoma growth is associated with an increase in the percentage of macrophages in the spleen as well as a decrease in the ability of tumor-bearer spleen cells to mount an antitumor cytotoxic response upon in vitro immunization. Here we provide evidence that macrophages in the MOPC-315 tumor-bearer spleen are responsible at least in part for the suppression of the generation of antitumor cytotoxicity. Accordingly, removal of most macrophages by depletion of phagocytic cells or Sephadex G-10-adherent cells from spleens of mice bearing a large tumor resulted in augmented antitumor immune potential. Also, Sephadex G-10-adherent spleen cells from tumor-bearing (but not normal) mice drastically suppressed the in vitro generation of antitumor cytotoxicity by normal spleen cells. The suppressive activity of these adherent cells did not reside in contaminating suppressor T cells, since it was not reduced by treatment with monoclonal anti-Thy 1.2 antibody plus complement. The Sephadex G-10-adherent cell population from the tumor-bearer spleen suppressed the in vitro generation of antitumor cytotoxicity against autochthonous tumor cells but not against allogeneic EL4 tumor cells, and hence the suppression was apparently specific. The suppressive activity of the Sephadex G-10-adherent cell population from tumor-bearer spleens was overcome by treatment of the tumor-bearing mice with a low curative dose of cyclophosphamide. This immunomodulatory effect of a low dose of the drug in overcoming the suppression mediated by the Sephadex G-10-adherent cell population enables the effector arm of the immune system of tumor-bearing mice to cooperate effectively with the drug's tumoricidal activity in tumor eradication.*

Introduction

Progression of tumor growth can be attributed to either inefficient in vivo sensitization or the appearance of suppressor elements that interfere with an effective antitumor immune response. The suppressor elements in tumor-bearing hosts described thus far include primarily blocking antibodies [12], free antigen shed by tumor cells [1], antigen-antibody complexes [13], T cells [9, 28, 35], macrophages [2, 10], and tumor cells [1, 26].

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In the MOPC-315 tumor model, we had shown previously [24, 26] that the progression of tumor growth is associated with a gradual reduction in the ability of spleen cells to mediate antitumor cytotoxicity upon in vitro immunization, and this correlates with the gradual increase in the percentage of metastatic tumor cells and of macrophages in the spleen. Partial recovery of the antitumor potential of tumor-bearer spleen cells was achieved by the depletion of tumor cells prior to in vitro immunization. Depletion of glass-adherent cells from tumor-bearer spleen cells removed most tumor cells and most macrophages and led to much greater augmentation of the antitumor potential than selective removal of tumor cells. Our results suggested that the antitumor cytotoxicity exhibited by tumor-bearer spleen cells is subject to suppression by macrophages [24, 26]. However, since glass wool removes not only the tumor cells and macrophages but also some lymphocytes, we could not be certain that the macrophages were indeed exerting suppressive activity.

The purpose of the work presented here is to demonstrate that the macrophages in the spleen of mice bearing a large MOPC-315 tumor are indeed responsible, at least in part, for the suppression of the antitumor immune potential of the spleen cells. In addition, we evaluated whether treatment of mice bearing a large tumor with a low curative dose of cyclophosphamide (CY) can overcome the immunosuppressive effect of the macrophages.

Materials and methods

Spleen cell suspensions. Spleen cell suspensions were prepared from normal female BALB/c mice (8–12 weeks old; Goodwin Institute for Cancer Research, Plantation, FL) or from mice bearing an SC MOPC-315 tumor. In any individual experiment performed, the spleens used in each group were obtained from at least three, but usually five to seven mice. Single-cell suspensions were prepared by mechanical disruption between glass slides as described previously [25], and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 95%.

Tumors. Tumor cells used in experiments were MOPC-315 plasmacytoma and EL4 leukemia. MOPC-315 was maintained by serial SC inoculation in syngeneic BALB/c mice. Single-cell suspensions were prepared by mechanical disruption [25], and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 85%. The C57BL/6 chemically

induced leukemia EL4 was adapted to culture in RPMI-1640 medium supplemented with 5% fetal calf serum, FCS, 50 U/ml penicillin, 50 μg/ml streptomycin (Grand Island Biological Co., Grand Island, NY). The EL4 cells were used as target or stimulator cells within 4 months following culture initiation.

In vitro immunization. The in vitro generation of antitumor cytotoxicity in lymphoid cells was done by a modification of the methods of Burton et al. [5] and Mokyr et al. [25]. Briefly, 40×10^6 responder spleen cells were cultured with 1.3×10^5 mitomycin C-treated MOPC-315 stimulator tumor cells in 30-ml tissue culture flasks (Falcon Plastic, Oxnard, CA) in a final volume of 20 ml RPMI-1640 medium supplemented with 1% nonessential aminoacids, 5% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin (Grand Island Biological Co., Grand Island, NY). Fresh medium was prepared on the day of the experiment and 2-mercaptoethanol (Sigma Chemical, Co., St Louis, MO) was added to give a final concentration of 5×10^{-5} *M* prior to use. The cultures were incubated at 37° C in 5% CO_2 in air for 5 days, the time required for the in vitro generation of optimal levels of antitumor cytotoxicity [25].

Antitumor cytotoxicity assay. Cell-mediated immune lysis was determined as described previously [25] by means of the ^{51}Cr-release assay [4]. Mixtures of 5×10^4 (0.4 ml) labeled target tumor cells and 5×10^6 spleen cells (0.4 ml) in 15- × 75-mm tubes were incubated at 37° C for 3.5 h. At the end of the incubation period cells were pelleted, and the supernatants were transferred to identical tubes. Both the supernatant and the pellet were counted in a gamma counter, and the percentage of ^{51}Cr release for each sample was calculated as follows.

$$\% \ ^{51}\text{Cr release} = \frac{\text{counts in supernatant}}{\text{counts in supernatant} + \text{counts in pellet}} \times 100 .$$

The percentage specific ^{51}Cr release was calculated by the following formula:

$$\% \text{ specific } ^{51}\text{Cr release} = \frac{T - S}{M - S} \times 100 .$$

where *T* is the percentage of lysis with test spleen cells; *S* is the percentage of spontaneous release (which was about 20% for MOPC-315 cells and about 15% for EL4 cells); and *M* is the percentage of maximal ^{51}Cr release (which was about 90% for MOPC-315 or EL4 cells) obtained from cells by three cycles of freezing and thawing. The data shown are representative of two to four individual experiments and the value for the percentage ^{51}Cr release is reported in the text as a numerical percentage representing the mean of triplicate samples. The variation in percentages of ^{51}Cr release between individual samples rarely exceeded 4% of the mean. All points differing by 6% ^{51}Cr release were judged to be significantly different ($P < 0.05$; Student's *t*-test).

Inactivation of macrophages. Selective inactivation of functional macrophages in vivo was done essentially by the method of Tracey [32]. Briefly, spleen cells were suspended in RPMI-1640 medium (in the absence of serum) to a concentration of 10^7 cells/ml and then incubated for 3 h with 100 μg silica (Monsanto Co., St Louis, MO) per milliliter.

Depletion of phagocytic cells by carbonyl iron and magnet. About 25 ml of a spleen cell suspension (2×10^7/ml) in RPMI-1640 medium containing 10% FCS was added to an equal volume of lymphocyte-separating reagent (Technicon Instruments Corp., Tarrytown, NY) containing carbonyl iron particles. The mixture was incubated with constant rocking at 37° C for 30 min. The cells that ingested carbonyl iron were removed by the use of a magnet according to the method described by Kirchner et al. [19]. To verify the effectiveness of the procedure in depleting macrophages, the percentage of macrophages was determined in the resultant nonphagocytic cell fraction and compared with the percentage of macrophages in the unseparated spleen cell suspension.

Depletion of Sephadex G-10-adherent spleen cells. Spleen cells were depleted of cells adhering to the column of Sephadex G-10 beads by the method of Ly and Mishell [23] with slight modification. Sephadex G-10 columns were prepared in the following manner. Sephadex G-10 was swollen overnight in three times its volume of distilled water. After swelling, the Sephadex G-10 beads were washed five times with Hanks' balanced salt solution (HBSS) in the same volume and then resuspended in twice the bed volume, and the slurry was boiled for 1–2 h. Sephadex G-10 thus prepared was stored at room temperature until used. Swollen Sephadex G-10 suspended in HBSS was pipetted into sterile 30-ml syringes plugged with six layers of gauze and approximately 2–3-ml glass beads (homogenizing, 0.25–0.30 mm, VWR Scientific Inc., Chicago, IL) and the Sephadex G-10 was allowed to settle by gravity until the bed volume was attained. Prior to use, the Sephadex G-10 column was equilibrated with RPMI-1640 medium containing 5% FCS. Before the spleen cells were applied to the column, red blood cells were lysed by treatment with an NH_4Cl-Tris buffer mixture. Then 2 ml of the spleen cell suspension ($1–1.5 \times 10^8$/ml) from normal or tumor-bearing mice were placed on the top of the Sephadex G-10 column and allowed to penetrate the column, after which 20–30 ml RPMI-1640 medium was immediately pipetted onto the column and the nonadherent cells were collected in the first 20 ml of the effluent. To verify the effectiveness of the fractionation in depleting macrophages, the percentage of macrophages in the resultant nonadherent spleen cell population was determined and compared with the percentage of macrophages in the unfractionated spleen cell suspension.

Determination of the percentage of macrophages. The percentage of macrophages in spleen cell suspensions of normal or tumor-bearing mice was determined by morphological and functional criteria. Enumeration of macrophages by morphological criteria was done by counting at least 2,500, but usually 5,000, nucleated cells on Wright stained smears. Enumeration of macrophages by functional criteria was done by determining the percentage of cells that (a) ingested latex beads (Difco Laboratories, Detroit, MI) according to the method of Rosenstreich et al. [29] and (b) stained red for esterase according to the method of Koshi et al. [20]. More than 90% of the cells identified as macrophages by morphological criteria phagocytized latex beads.

Collection of Sephadex G-10-adherent spleen cells. Sephadex G-10-adherent cells were collected by the method of Chang et al. [6]. Briefly, the above column was washed with 150–200 ml RPMI-1640 medium supplemented with 5% FCS until the effluent was almost free of cells ($< 5 \times 10^4$ cells/ml). Adherent

cells were harvested by incubating the column with 30 m*M* Lidocaine (Sigma Chemical Co., St Louis, MO) solution in RPMI-1640 medium containing 20% FCS, at 37° C in a 5% CO_2 atmosphere for 20 min. Following incubation, adherent cells were harvested by rapidly eluting the column with 50–80 ml RPMI-1640 medium supplemented with 5% FCS. Of the original number of cells applied to the column, approximately 4% of normal or 10% of tumor-bearer spleen cells were recovered as adherent cells from the Sephadex G-10 column. The viability of these adherent cells as determined by trypan blue dye exclusion always exceeded 85%. The adherent spleen cell population was evaluated for cell composition. The macrophages were the predominant cells in the adherent fraction (approximately 70%); however, cells bearing Thy 1.2 antigens and cells bearing surface immunoglobulin were also present ($\leq$ 10% and $\leq$ 30%, respectively). Enumeration of Thy 1.2-positive cells and immunoglobulin-positive cells was done by fluorescence microscopy. Thy 1.2-positive cells were identified through the use of FITC-coupled avidin (Sigma Chemical Corporation, St Louis, MO) plus biotinylated rat anti-Thy 1.2 monoclonal antibody, while immunoglobulin-positive cells were identified through the use of FITC-coupled avidin plus biotinylated rabbit antimouse IgG.

Isolation of plastic-adherent spleen cells. To obtain plastic-adherent cells, 20 ml of a suspension of spleen cells containing 10×10^6 cells/ml in minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 20% FCS was aliquoted onto a 150 × 25 mm tissue culture plate (Falcon Plastics, Oxnard, CA) and incubated at 37° C in 5% CO_2 in air. The plates were washed twice with the same prewarmed medium 6 h later, and incubated further with the same medium. After 12–18 h, the plates were washed four times and adherent cells were obtained by mechanical dislodging with a rubber policeman.

Depletion of T cells from adherent tumor-bearer spleen cells by anti-Thy 1.2 antibody and complement. Sephadex G-10-adherent cells (1 ml; 2×10^7/ml) were mixed with an appropriate volume of monoclonal anti-Thy 1.2 antibody (30-H12; Ledbetter and Herzenberg [22]) to give a final dilution of 1 : 10. The cells were incubated at 4° C for 45 min, and then mixed with an appropriate volume of complement (guinea pig serum) to give a final dilution of 1 : 6. After a further incubation at 37° C for 30 min the cells were washed three times and resuspended in RPMI-1640 supplemented with 5% FCS before addition to the immunization culture of normal spleen cells. The viability of the adherent cells thus treated exceeded 80%. This treatment reduced the percentage of Thy 1.2-positive cells in the Sephadex G-10-adherent spleen cell fraction from approximately 10% to less than 0.3%. The effectiveness of the protocol in depleting T cells was also illustrated by the ability of the monoclonal anti-Thy 1.2 antibody and complement to abolish the ability of spleen cells to generate T cell-dependent antitumor immunity, and to proliferate in response to a T-cell mitogen, phytohemagglutinin.

Chemotherapy. CY (Cytoxan; Mead Johnson and Co., Evansville, IN) was dissolved in sterile distilled water (to a concentration of 20 mg/ml) and further diluted in minimum essential medium. CY therapy consisted of a single IP injection of 15 mg/kg.

Results

Augmentation of the antitumor immune potential of MOPC-315 tumor-bearer spleen cells by depletion of phagocytic or Sephadex G-10-adherent cells or by inactivation of phagocytic cells

We have previously observed that progression of tumor growth is associated with an increase in the percentage of macrophages in the spleen as well as a decrease in the level of antitumor cytotoxicity obtained upon in vitro immunization [24, 26]. Therefore, experiments were performed to determine whether removal of most macrophages by depletion of phagocytic or Sephadex G-10-adherent cells from spleens of tumor-bearing mice would lead to augmented antitumor cytotoxicity upon in vitro immunization (Tables 1 and 2). Spleen cells from mice bearing a 16-mm tumor contained 17% macrophages and upon in vitro immunization they exhibited a lower level of antitumor cytotoxicity than that exhibited by in vitro-immunized spleen cells from normal mice (7% vs 85%) (Table 1). Treatment of the tumor-bearer spleen cells with carbonyl iron and magnet reduced the percentage of phagocytic cells (macrophages) to about 2% and upon in vitro immunization it resulted in an augmented level of antitumor cytotoxicity (38% vs 7%) (Table 1).

Depletion of Sephadex G-10-adherent cells from tumor-bearer spleen cells also augmented their antitumor immune potential (Table 2). Spleen cells from mice bearing a 26-mm tumor contained 21% macrophages and upon in vitro immunization exhibited no cytotoxicity, whereas a substantial level of cytotoxicity (50%) was exhibited by in vitro-immunized spleen cells of normal mice. Fractionation of the tumor-bearer spleen cells by a Sephadex G-10 column removed most of the macrophages (from 21% to 2%) and upon in vitro immunization it resulted in an augmented level of antitumor cytotoxicity (30%).

Since removal of phagocytic or adherent cell populations from the spleen may also remove other cells, immunosuppressed tumor-bearer spleen cells were incubated in vitro with silica to inactivate the phagocytic cells (Table 3). Incubation of the immunosuppressed tumor-bearer spleen cells with silica resulted in a partial restoration of their antitumor immune potential. Accordingly, upon in vitro immunization, the silica-treated tumor-bearer spleen cells exhibited an augmented antitumor cytotoxic activity compared with that of untreated tumor-bearer spleen cells (27% vs 13%). Thus, the reduced ability of spleen cells from mice bearing a large tumor

Table 1. Effect of depletion of phagocytic cells from MOPC-315 tumor-bearer spleen cells on the level of antitumor cytotoxicity generated upon in vitro immunization with MOPC-315 stimulator tumor cells

Source of responder spleen cells	Depletion of phagocytic cells by carbonyl iron and magnet	Percent macrophages in pooled spleen cells	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE)[a]
Normal mice	–	3.6	85.2 ± 2.5
Tumor bearer[b]	–	16.7	7.0 ± 0.2
Tumor bearer[b]	+	2.1	38.1 ± 0.2

[a] At an effector: target cell ratio of 100: 1
[b] Mice bearing a tumor 16.1 ± 0.8 mm in diameter

Table 2. Effect of depletion of Sephadex G-10-adherent cells from MOPC-315 tumor-bearer spleen cells on the level of antitumor cytotoxicity generated upon in vitro immunization with MOPC-315 stimulator tumor cells

Source of responder spleen cells	Depletion of adherent cells by use of Sephadex G-10	Percent macrophages in pooled spleen cells	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE) at an effector: target cell ratio of	
			50:1	20:1
Normal mice	–	3.3	49.6 ± 1.0	23.3 ± 0.7
Tumor bearer[a]	–	20.6	− 8.1 ± 0.2	− 4.0 ± 0.2
Tumor bearer[a]	+	1.9	29.9 ± 0.1	9.0 ± 0.4
Normal mice	+	1.0	24.4 ± 0.3	4.0 ± 0.9

[a] Mice bearing a tumor 26.0 ± 1.0 mm in diameter

Table 3. Effect of inactivating phagocytic cells in MOPC-315 tumor-bearer spleen cells on the level of antitumor cytotoxicity obtained upon in vitro immunization

Source of responder spleen cells	Treatment with silica	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE) at an effector: target cell ratio of	
		100:1	20:1
Normal mice	–	45.4 ± 1.0	16.4 ± 1.2
Tumor bearer[a]	–	12.7 ± 0.2	4.7 ± 0.4
Tumor bearer	+	26.7 ± 0.6	12.1 ± 1.1

[a] Mice bearing a tumor 23.0 ± 0.8 mm in diameter

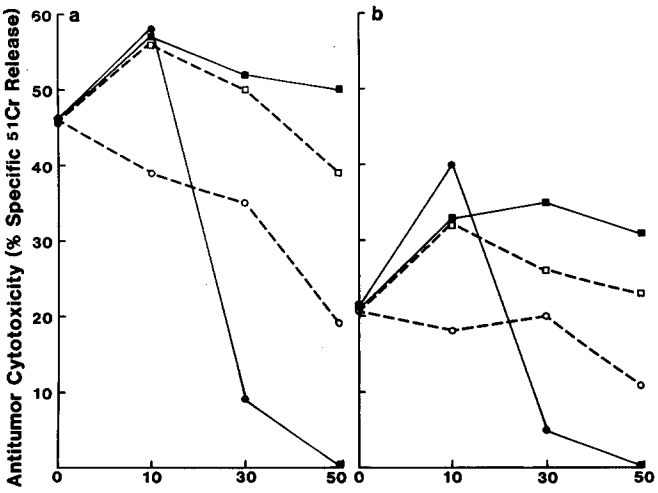

Fig. 1a and b. Suppression of the antitumor immune potential of normal spleen cells by addition of Sephadex G-10-adherent cells from spleens of normal mice or mice bearing a 31-mm tumor. The adherent cells were added so as to constitute 10%, 30%, or 50% of the cell population, and following in vitro immunization these cell populations were assessed for antitumor cytotoxicity by the ^{51}Cr-release assay. The cytotoxicity assay is shown for an effector: target cell ratio of 50:1 **(a)** and 20:1 **(b)**. ●, adherent cells from the tumor-bearer spleen; ○, adherent cells from the normal spleen. ■, nonadherent cells from the tumor-bearer spleen; □, nonadherent cells from the normal spleen

to mediate antitumor cytotoxicity upon in vitro immunization is due at least in part to the suppressive activity of cells which are phagocytic and adhere to Sephadex G-10.

Suppression of the antitumor immune potential of normal spleen cells by addition of adherent cells from tumor-bearer spleens

Different numbers of Sephadex G-10-adherent spleen cells from normal mice or mice bearing a 31-mm tumor were added to normal spleen cell cultures so as to constitute 10%, 30%, or 50% of the total cell population, and subsequently the spleen cells were immunized in vitro (Fig. 1). The presence of 10% adherent cells from the tumor-bearer spleen among normal spleen cells prior to in vitro immunization resulted in a slight augmentation in the level of antitumor cytotoxicity (58% vs 46%). However, the presence of 30% or 50% adherent cells from the tumor-bearer spleen reduced the level of antitumor cytotoxicity (from 46% to 9% or 0.3%, respectively). In a control experiment, the presence of 30% or 50% Sephadex G-10-adherent cells from normal spleens also reduced the level of antitumor cytotoxicity (from 46% to 35% or 19%), but this suppression was much less than that obtained by the adherent cells of tumor-bearer spleens ($P < 0.01$). On the other hand, the *nonadherent* cells from spleens of either normal or tumor-bearing mice did not suppress the levels of antitumor cytotoxicity (Fig. 1). This result indicated not only that the nonadherent cells were not immunosuppressive but also that the immunosuppressive effect of the adherent cells was not due to deficient culture conditions. Finally, when plastic-adherent cells were used instead of Sephadex G-10-adherent cells, the presence of 30% adherent cells from the spleen of a mouse bearing a 30-mm tumor also reduced the level of antitumor cytotoxicity (from 78% to 30%).

Thus, the *adherent* but not the *nonadherent* cells of tumor-bearer spleens suppress the antitumor response of normal spleen cells. Moreover, the *adherent* cells from a tumor-bearer spleen are much more suppressive than those from a normal spleen.

Immunosuppressive activity of Sephadex G-10-adherent spleen cells with progression of tumor growth

Sephadex G-10-adherent spleen cells were obtained from mice bearing a 20-, 25-, or 30-mm tumor. The isolated adherent cells were added to normal spleen cells so as to constitute 30% of the total cell population and subsequently the cell mixture was

Table 4. Effect of treating Sephadex G-10-adherent, tumor-bearer spleen cells with anti-Thy 1.2 antibody plus complement (C) on their ability to suppress the in vitro generation of antitumor cytotoxicity by normal spleen cells

Percentage of the added adherent tumor-bearer spleen cells	Treatment with anti-Thy 1.2 plus C[a]	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE) at an effector: target cell ratio of	
		50:1	20:1
0	−	24.6 ± 0.2	11.8 ± 0.4
30	−	12.6 ± 0.5 (48.8%)[b]	5.5 ± 0.7 (53.4%)[b]
30	+	11.1 ± 0.4 (54.9%)[b]	5.2 ± 0.5 (55.9%)[b]

[a] Neither anti-Thy 1.2 antibody nor C (complement) alone was toxic for spleen cells
[b] Percent inhibition of the cytotoxic response is indicated in parentheses

Table 5. Specificity of suppression by normal adherent spleen cells of the generation of antitumor cytotoxicity by normal spleen cells

Stimulator tumor cells	Percentage of the added normal adherent spleen cells	Target cells	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE) at an E:T cell ratio of	
			100:1	20:1
MOPC-315	0	MOPC-315	59.8 ± 2.3	39.5 ± 0.8
MOPC-315	30	MOPC-315	42.4 ± 0.5 (29.1%)[a]	27.8 ± 0.6 (29.6%)[a]
EL4	0	EL4	85.9 ± 0.3	52.8 ± 4.0
EL4	30	EL4	67.7 ± 0.2 (21.2%)[a]	31.6 ± 1.2 (40.2%)[a]

[a] Percent inhibition of specific ^{51}Cr release is indicated in parentheses

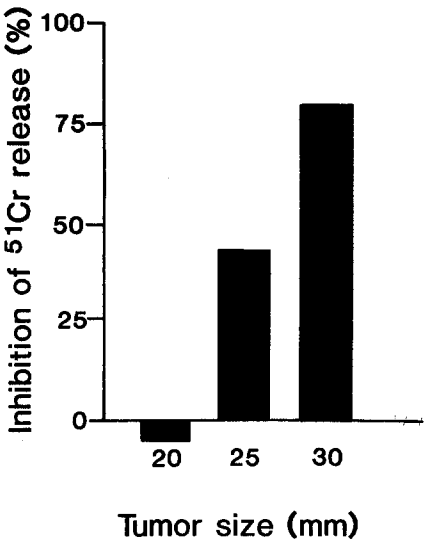

Fig. 2. Increase in the immunosuppressive activity of Sephadex G-10-adherent spleen cells with progression of tumor growth. Adherent cells from mice bearing a 20-, 25-, or 30-mm tumor were added to normal spleen cells so as to constitute 30% of the cell population. Following in vitro immunization these cell populations were assessed for antitumor cytotoxic activity by the ^{51}Cr-release assay and the percent inhibition was calculated

immunized in vitro (Fig. 2). No suppression was obtained with adherent cells from mice bearing a 20-mm tumor, substantial suppression was obtained with adherent cells from mice bearing a 25-mm tumor (42%), and almost complete suppression occurred with adherent cells from mice bearing a 30-mm tumor (81%). Thus, the immunosuppressive activity of the adherent spleen cells increases with progression of tumor growth.

The role of T cells in the immunosuppressive activity of Sephadex G-10-adherent, tumor-bearer spleen cells

To rule out the possibility that the immunosuppressive activity of Sephadex G-10-adherent, tumor-bearer spleen cells might be due to the action of suppressor T cells present as contaminants in the eluted adherent cell fraction, we evaluated the effect of eliminating T cells on the immunosuppressive activity of the adherent cells. This was done by treating the Sephadex G-10-adherent cells with monoclonal anti-Thy 1.2 antibody plus complement, adding them to normal spleen cells to constitute 30% of the cell population, and subsequently immunizing the cell mixture in vitro (Table 4). The ability of the splenic adherent cells from tumor bearers to suppress the generation of antitumor cytotoxicity was not altered following treatment with anti-Thy 1.2 antibody plus complement, i.e., the suppression of the cytotoxic response was 55% for the treated compared with 49% for the untreated adherent cells. Similarly, anti-Thy 1.2 antibody plus complement did not alter the immunosuppressive activity of splenic adherent cells from normal mice (data not shown).

Evaluation of the specificity of immunosuppression mediated by tumor-bearer or normal adherent spleen cells on the generation of antitumor cytotoxicity by normal spleen cells

Sephadex G-10-adherent spleen cells from normal BALB/c mice or BALB/c mice bearing a 30-mm MOPC-315 tumor were added to normal BALB/c spleen cell cultures so as to constitute 30% of the total cell population. The cell mixture was immunized in vitro with syngeneic MOPC-315 or allogeneic EL4-stimulator tumor cells and subsequently assayed for antitumor cytotoxicity against MOPC-315 or EL4 target cells by the ^{51}Cr-release assay (Tables 5 and 6). The presence of 30% adherent cells from the *normal* BALB/c spleen slightly

Table 6. Specificity of suppression by tumor-bearer adherent spleen cells of the generation of antitumor cytotoxicity by normal spleen cells

Stimulator tumor cells	Percentage of the added tumor-bearer adherent spleen cells	Target cells	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE) at an E : T cell ratio of	
			50:1	20:1
MOPC-315	0	MOPC-315	30.0 ± 0.7	12.7 ± 0.7
MOPC-315	30	MOPC-315	1.9 ± 0.2 (93.7%)[a]	1.9 ± 1.9 (85.0%)[a]
EL4	0	EL4	69.2 ± 2.4	54.5 ± 1.3
EL4	30	EL4	58.1 ± 1.5 (16.0%)[a]	39.7 ± 2.6 (27.2%)[a]

[a] Percent inhibition of specific ^{51}Cr release is indicated in parentheses

Table 7. Effect of cyclophosphamide therapy (15 mg/kg) of tumor-bearing mice on the suppressive activity of their Sephadex G-10-adherent cells for the in vitro generation of antitumor cytotoxicity by normal spleen cells

Source of adherent spleen cells	CY therapy[a]	Percentage of the added adherent cells	Antitumor cytotoxicity (% specific ^{51}Cr release ± SE) at an effector: target cell ratio of	
			50:1	20:1
None	–	0	33.9 ± 0.7	27.5 ± 0.2
Normal mice	–	30	30.8 ± 0.2	28.5 ± 0.2
Tumor bearers[b]	–	30	23.5 ± 0.6	23.8 ± 0.8
Tumor bearers[b]	+	10	74.1 ± 1.4	77.4 ± 1.0
Tumor bearers[b]	+	30	77.4 ± 1.5	76.3 ± 1.4
Tumor bearers[b]	+	50	70.7 ± 3.8	69.6 ± 2.3

[a] The adherent spleen cells were obtained 10 days after CY therapy with 15 mg/kg
[b] Mice bearing a tumor 30 ± 1.2 mm in diameter

inhibited the generation of cytotoxicity against MOPC-315 or EL4 tumor cells to about the same extent (29% and 21%, respectively) (Table 5). However, the presence of 30% adherent cells from the *tumor-bearer* BALB/c spleen drastically inhibited the generation of anti-MOPC-315 cytotoxicity (94%) but only slightly inhibited the generation of anti-EL4 cytotoxicity (16%) (Table 6). Thus, although the immunosuppression mediated by tumor-bearer adherent spleen cells was partly nonspecific, it appeared to be primarily specific against autochthonous tumor cells.

Evaluation of the suppressive ability of Sephadex G-10-adherent cells obtained from spleens of mice cured of a large tumor with a low dose of cyclophosphamide

Mice bearing a 30-mm tumor were treated with CY, 15 mg/kg, and 10 days later, the Sephadex G-10-adherent spleen cells were obtained and evaluated for their suppressive activity. The immunosuppressive activity was evaluated by adding adherent cells to normal spleen cells so as to constitute 10%, 30%, or 50% of the total population and subsequently the cell mixture was immunized in vitro (Table 7). In contrast to the adherent cells from untreated tumor bearers, which were immunosuppressive (23% compared with the control value of 34%), the adherent cells from CY-treated tumor bearers did not immunosuppress but actually enhanced the generation of antitumor immunity (> 70% compared with the control value of 34%).

Discussion

Spleen cells of mice bearing a large SC MOPC-315 tumor exhibit a reduced ability to mount an antitumor cytotoxic response upon in vitro immunization [25]. Here we show that this reduced antitumor immune potential is due at least in part to the suppressive activity of macrophages. The evidence that macrophages are indeed immunosuppressive in mice bearing a large MOPC-315 tumor is as follows. When macrophages were depleted from tumor-bearer spleens by their property of adherence to Sephadex G-10 or phagocytosis of carbonyl iron, the percentage of macrophages was reduced from about 19% to less than 2% and both of these procedures resulted in a substantial augmentation of the antitumor immune potential. When immunosuppressed tumor-bearer spleen cells were treated with silica to inactivate the macrophages, this resulted in partial restoration of the antitumor immune potential of the spleen cells. We know from our previous work [26] that the immunosuppression in the MOPC-315 tumor-bearer spleen is due in part to metastatic tumor cells and possibly suppressor T lymphocytes. However, it is highly unlikely that all three procedures known to deplete or inactivate macrophages also eliminated the immunosuppressive activity of another cell type. Indeed, treatment of tumor cells with carbonyl iron and magnet did not reduce the ability of viable tumor cells to suppress the in vitro generation of antitumor immunity (data not shown). To rule out the possibility that contaminating T cells are the cause of the observed immunosuppression, we eliminated them from the Sephadex G-10-adherent cell population by treatment with monoclonal anti-Thy 1.2 anti-

body and complement prior to the addition of the adherent cell population to normal spleen cells. The immunosuppression mediated by Sephadex G-10-adherent cells from the spleen of MOPC-315 tumor-bearing mice was apparently specific and was overcome by a low curative dose of CY.

Several reports implicate the macrophage in the spleen of the tumor-bearing host as responsible for the suppression of the host's cell-mediated immunity. In these studies, macrophages derived from tumor bearers suppressed the proliferative response of lymphoid cells to mitogens [8, 27] and tumor-associated antigens [11] as well as the cytotoxic response of lymphoid cells to allogeneic lymphoid cells [10]. Here we extend these observations by showing that the generation of the cytotoxic response of lymphoid cells to autochthonous tumor cells is also suppressed by macrophages.

The increase in the suppressive activity of macrophages in the tumor bearer has been attributed to an excess number of macrophages [21, 34] or to a suppressor function induced during tumor growth [2]. Bluestone and Lopez [2] have shown that the immunosuppressive activity of tumor-bearer macrophages was not due merely to the increased percentage of macrophages, since a relatively low percentage of splenic macrophages of tumor-bearing mice but not of normal mice suppressed the mixed lymphocyte reaction. We evaluated the ability of splenic macrophages from tumor bearers to suppress the in vitro generation of antitumor cytotoxicity by normal spleen cells, and compared it with the immunosuppressive ability of splenic macrophages from normal mice. Although splenic macrophages from either normal or tumor-bearing mice suppressed the in vitro generation of antitumor cytotoxicity, the suppressive activity induced by the macrophages of the tumor bearers was much greater and occurred with a smaller percentage of cells. Moreover, splenic macrophages of the tumor bearers differed from splenic macrophages of normal mice in the specificity of their immunosuppression. The tumor-bearer macrophages drastically suppressed the in vitro generation of antitumor cytotoxicity against autochthonous tumor cells, but to a much lesser extent against allogeneic tumor cells. In contrast, the suppression mediated by normal macrophages was equally suppressive for the generation of antitumor cytotoxicity against syngeneic or allogeneic tumor cells. Thus, our results demonstrate that splenic macrophages of mice bearing a large MOPC-315 tumor are qualitatively different than normal splenic macrophages in their immunosuppressive activity.

According to our previous observations [14], injection of a low dose of CY into mice bearing a large tumor resulted in an augmented ability of their spleen cells to mount an antitumor cytotoxic response upon in vitro immunization. This augmentation was accompanied by the elimination of tumor cells in the spleen within 2 days, yet the macrophage population remained elevated for at least 7 days. Here we demonstrate that the Sephadex G-10-adherent spleen cells from mice cured with a low dose of CY, even when present as 50% of the cell population, did not suppress but actually augmented the antitumor potential of normal spleen cells. Although this result suggests that the CY therapy leads to the elimination of suppressor macrophages, the possibility remains that the suppressive activity of macrophages may be masked by the augmented activity of other Sephadex G-10-adherent cells.

Several investigators have attempted to elucidate the mechanism of induction of suppressor macrophages during progression of tumor growth [18, 30]. In these studies, the suppressive activity of normal macrophages was greatly enhanced by exposure to tumor cells or tumor cell products. Kenmarch and Zolla-Pozner [18] demonstrated that plasmacytomas produce a factor (the PC factor) that can induce normal peritoneal macrophages to produce an immunosuppressive molecule (plasmacytoma-induced macrophage substance, PIMS) which is different from thymidine or prostaglandin and which is capable of suppressing the primary antibody response in vitro. Moreover, they found that the PIMS is produced by macrophages from the spleen of plasmacytoma-bearing mice [18], thus indicating that this is a mechanism for the induction of suppressor macrophages during progression of plasmacytoma growth. Another possible mechanism is suggested by the work of Ting and Hargrove [30], who demonstrated that the interaction between FBL-3 tumor cells and macrophages triggers the production of prostaglandins, which in turn induces the generation of suppressor T cells. This tumor-induced, macrophage-mediated immunosuppression was nonspecific [30], as was that reported by Kenmarch and Zolla-Pozner [18].

Although our results demonstrate that Sephadex G-10-adherent cells from spleens of MOPC-315 tumor-bearing mice drastically suppress the generation of antitumor cytotoxicity against autochthonous tumor cells and to a much lesser extent against allogeneic EL4 tumor cells, the degree of specificity of this macrophage-mediated suppression has to be vigorously tested against a panel of tumors (i.e., plasmacytoma or nonplasmacytoma of BALB/c as well as non-BALB/c origin). If specificity of the suppression is then established, it could occur as a result of preferential accumulation of specific T cells near the macrophages through some form of recognition system. This seems plausible in view of the recent reports by Hoover et al. [15, 16] which show that progression of MOPC-315 tumor growth is accompanied by an increase in regulatory T cells with Fc_{α} receptors. Thus, it is not unreasonable to expect that anti-immunoglobulin idiotype complexes associated with regulatory T cells might serve to concentrate them near macrophages and thereby lead to the induction of specific suppressor T-cell activity.

Peripheral blood mononuclear phagocytes have been shown to be responsible at least in part for the impaired immune reactivity of lymphoid cells from patients with different types of cancer, including Hodgkin's lymphoma, melanoma, bladder cancer, multiple myeloma, breast cancer, and lung cancer [3, 7, 17, 33]. Of particular interest is the study by Dean et al. [7] demonstrating that following surgery for stage 1 lung cancer, patients with suppressed immune responsiveness (following PHA stimulation or in mixed lymphocyte cultures) suffer recurrent disease earlier and more frequently than do patients with normal immune responsiveness. The recrudescence was attributed to persistent elevated monocyte-mediated suppression of the antitumor immunity needed for the eradication of residual tumor cells. In light of our results, it might be possible to decrease the frequency of recurrence of disease in such patients by administering a low dose of CY to overcome monocyte-mediated immunosuppression.

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