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Specific Depression of the Antitumor Cellular Immune Response with Autologous Tumor Homogenate

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SUMMARY

A homogenate of an SV40-transformed fibrosarcoma of BALB/c mice (E_4 tumor) injected i.p. into E_4 tumor-immune syngeneic mice specifically depressed their cell-mediated immune responses to autologous tumor cells, as measured by a radioisotopic foot pad assay. The fraction of the tumor homogenate that brought about this depression was present in the high-speed supernatant and pellet of a 3 M KCl extract of the tumor. The specificity of the depression was shown in three ways: (*a*) the serum of E_4 tumor-immune mice, but not of normal mice, given injections of E_4 tumor homogenate 24 hr previously, suppressed antitumor immunity *in vitro*, as measured by the release of ^{51}Cr from labeled E_4 tumor cells incubated with spleen cells from tumor-immune animals; (*b*) the i.p. inoculation of E_4 tumor homogenate did not alter the cellular immune response of tuberculin-sensitized mice to tuberculin; and (*c*) the i.p. injection of a homogenate of antigenically unrelated tumor did not depress the cellular immune response of E_4 tumor-immune mice to E_4 tumor cells.

INTRODUCTION

It has been extensively demonstrated that a host is capable of developing a specific immune response directed against a tumor growing *in situ* (3, 5, 6, 11, 16). Recent work has extended these findings, showing that, at least in certain experimental tumor systems, a definite relationship exists between tumor size and the magnitude of the antitumor immune response (5, 12, 21). This response is "eclipsed" or specifically suppressed to nondetectable levels as the tumor continues to grow larger. We showed previously that the eclipse mechanism in tumor-bearing animals does not involve the elimination of specifically reactive immunocytes: lymphocytes from tumor-bearing mice were immunocompetent in a local adoptive transfer reaction against the tumor in normal syngeneic animals (12). Since lymphocytes in animals with large tumors are nonreactive in the host, it has been postulated that a factor or factors derived from the tumor may be responsible, in part, for the observed immunological suppression. The fact that a tumor-bearing animal recovers from the eclipsed state after tumor excision supports this view (3, 4, 8, 12). Some investigators have suggested that the "blocking factor(s)" that suppress antitumor immunity are antigen-antibody complexes (2, 14); however, other possibilities exist (5, 17, 19, 20). Our present studies were undertaken to determine the source and characteristics of the factor(s) responsible for this eclipse phenomenon in the SV40-transformed tumor murine host system.

The experiments reported here demonstrate that a homogenate of material from SV40-transformed tumors injected i.p. into tumor-immune mice was capable of suppressing their cell-mediated response to the same tumor. The ability to lower this response was also present in a 3 M KCl extract of the tumor. Cell-mediated immunity was measured *in vivo* by the radioisotopic footpad assay. Serum of mice inoculated with this tumor homogenate suppressed immune lymphocyte reactivity *in vitro* as measured by the release of ^{51}Cr from the labeled tumor cells. This immune depression was specific, as cellular immunity was not impaired by injection of an antigenically unrelated syngeneic methylcholanthrene-induced tumor.

MATERIALS AND METHODS

Animals and Cell Lines. Six- to 8-week-old male BALB/c AnN mice, obtained from the Animal Production unit, NIH, Bethesda, Md., were used throughout these experiments.

The E_4 cell line (10) was started from an explant of solid fibrosarcoma in a BALB/c mouse produced by inoculating it with SV40-transformed BALB 3T3 cells. The methylcholanthrene tumor line (MCA-15) was started from a solid fibrosarcoma that developed in a BALB/c mouse 3 months after the s.c. injection of 0.2 mg of methylcholanthrene in Mazola oil. Both cell lines were carried in Dulbecco Vogt's modification of Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Production of E_4 and MCA-15 Tumors Used as a Source of Tumor Homogenate. BALB/c mice were given injections of 1×10^6 E_4 or MCA-15 cells in the right flank, and the resulting tumors were excised 3 to 4 weeks later. The average tumor weight was 2.0 g.

Protocol for Obtaining a Crude Homogenate and Soluble Supernatant from Tumor Material. Tumor material was thoroughly homogenized in 2 volumes of cold PBS[1] with a mortar and pestle. A small aliquot of homogenate was observed microscopically to ensure that all the cells were completely disrupted.

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[1] The abbreviation used is: PBS, phosphate-buffered saline (0.01 M phosphate in 0.85% NaCl).

A homogenate of material from a MCA-15 tumor was obtained in the same manner. The tumor homogenates were centrifuged for 60 min at 20,000 rpm (S-40 rotor) in a Model L ultracentrifuge. The soluble supernatant (excluding a thin lipid layer at the air-liquid interface) was carefully removed. The protein content was determined by Lowry's technique.

Preparation of 3 M KCl Extract of the E_4 Tumor. Twenty-five g of finely minced E_4 tumor were extracted at 37° for 30 min with 10 volumes of 3 M KCl in 0.05 M potassium phosphate buffer, pH 7.0, with continuous stirring. The slurry was centrifuged at 10,000 rpm for 30 min. The pellet (labeled Pellet 1) was washed 3 times with 5 volumes of PBS (suspension was centrifuged at 3,000 rpm for 15 min in a PR-J centrifuge). The supernatant was dialyzed against 4 liters of distilled water for nearly 24 hr in the cold. Distilled water was changed once during this period of dialysis. Later the dialysate was centrifuged at 95,000 × *g* for 1 h. The pellet was labeled Pellet 2. The supernatant was concentrated to 10.0 ml with Sephadex 150 and designated as Supernatant 2 (Pharmacia, Uppsala, Sweden). To each ml of concentrated Supernatant 2, 0.05 ml 17% NaCl was added to make it isotonic. Pellets 1 and 2 were separately resuspended in PBS and then sonically extracted in a Raytheon Model DF101 sonic oscillator (Raytheon Co., N. H.) for 2 to 3 min. The protein content was determined by Lowry's technique (10).

Immunization of Animals. E_4 tumor-immune mice were routinely produced by excising tumors from E_4 tumor-bearing mice and subsequently challenging them 10 days later with 1×10^6 syngeneic tumor cells. Mice that rejected this challenge were considered immune (11).

Tuberculin-sensitized animals were obtained by giving mice s.c. injections of 0.5 ml of complete Freund's adjuvant (*Mycobacterium tuberculosis* H37 RA; Difco Laboratories, Detroit, Mich., in the groin. The inoculations were repeated after 3 weeks.

Cellular Immune Response of Immune Mice Given Injections of Tumor Homogenate. E_4-immune mice, tuberculin-immune mice, or normal mice were given i.p. injections of 0.20 to 0.25 g (wet weight) of E_4 or MCA-15 tumor homogenate according to protocol. Twenty-four hr later the mice were given injections in the footpad with the challenge dose of either 1×10^6 E_4 tumor cells or with *M. tuberculosis* extract containing 10 μg of protein. The method of preparation of this extract has been described previously (11). The immune response was measured by the radioisotopic footpad assay (11). Briefly, following inoculation of the footpad, the mice are given i.p. injections of ^{125}I-labeled mouse serum protein. The increased capillary permeability associated with the cell-mediated immune reaction allows the labeled protein to leak into the interstitial fluid and accumulate at the site of the injection. This results in an increased number of counts in the challenged footpad as compared to the normal footpad. Twenty-four hr after challenge, the test footpad and the contralateral footpad are cut off and counted in a γ spectrometer. Results are expressed as a ratio of counts in the test foot to those in the control foot (foot count ratio). Control groups consisted of immune mice not given injections of homogenates of tumor material and challenged with E_4 tumor cells or *M. tuberculosis* extract in the footpad.

In other experiments, 1.0 ml of soluble supernatant fluid, obtained by centrifugation of homogenized tumors, was injected i.v. or i.p. into tumor-immune mice; 24 hr later the mice were challenged with tumor cells in the footpad as described.

i.p. Inoculation of E_4 Tumor-immune Mice with 3 M KCl Extract of E_4 Tumor. E_4 immune or normal mice were given i.p. injections of either Pellet 1, Pellet 2, or Supernatant 2. Twenty-four hr later the cellular immune response was measured by the radioisotopic footpad assay. Control groups not given i.p. injections of tumor material were also included in the study. Each mouse was given injections of aliquots of Supernatant 2 and Pellet 2 containing 0.45 and 0.85 mg of protein, respectively. Aliquots of Pellet 1 injected per mouse contained 10 mg of protein.

Antigenicity of Soluble Supernatant of Tumor Homogenate. A soluble supernatant containing 3.0 mg of protein was injected into footpads of E_4 tumor-immune and normal mice. The cellular immune response was measured by the radioisotopic footpad assay. Results were statistically analyzed by the Student *t* test.

***In Vitro* Cytotoxicity Assay to Measure Release of ^{51}Cr from E_4 Tumor Cells.** E_4 tumor cells (1×10^8) were labeled by incubating them with 50 μCi ^{51}Cr for 30 min at 37°. The cells were washed twice before use. The incubation mixture consisted of 3×10^6 (sensitized or normal) spleen cells and 5×10^5 labeled E_4 tumor cells (1). Serum harvested from normal and tumor-immune mice previously given injections of tumor homogenates was added to the appropriate cells to a final concentration of 10%. After incubation for 48 hr, the cells were centrifuged and the pellet and supernatant were counted separately. A pellet of 5×10^5 labeled E_4 tumor cells was frozen and thawed 3 times to determine total uptake of ^{51}Cr. Spontaneous release of ^{51}Cr from labeled E_4 tumor cells into the supernatant was measured by incubating 5×10^5 labeled E_4 tumor cells with 3×10^6 unlabeled E_4 tumor cells. There was no significant difference between ^{51}Cr uptake and spontaneous release by E_4 tumor cells alone and uptake and release of ^{51}Cr of tumor cells incubated with normal spleen cells.

RESULTS

Specific Suppression of the Antitumor Cellular Immune Response Produced by the i.p. Inoculation of Tumor Homogenates. A group of E_4 tumor-immune mice received an i.p. injection of 0.25 g (wet weight) of homogenized material from homologous tumor. Twenty-four hr later the mice were challenged with 1×10^6 E_4 tumor cells injected into the footpad, and the cell-mediated immune response was measured by the radioisotopic footpad assay. The results in Table 1 show that, while untreated E_4-immune mice gave a significant foot count ratio of 2.83 ± 0.35 (Group 3), the group of E_4-immune animals previously given injections of 0.20 to 0.25 g of tumor homogenate (Group 1) gave a foot count ratio of 1.22 ± 0.07 ($p < 0.001$)

Table 1

Specific depression of the cellular immune response in E_4 tumor-immune mice 24 hr after i.p. injection of tumor homogenate

Group	Source of tumor homogenate material injected i.p.	Status of recipient mice[a]	Foot count ratio (after challenge with 1×10^6 E_4 tumor cells)[b]
1	E_4 tumors	E_4 tumor-immune	1.22 ± 0.07[c]
2	MCA-15 tumors	E_4 tumor-immune	2.74 ± 0.17
3	No i.p. injection	E_4 tumor-immune	2.83 ± 0.35
4	No i.p. injection	Normal	1.46 ± 0.11

[a] Ten mice per group.
[b] Group 1 *versus* Group 3, $p < 0.001$.
[c] Mean ± S.E.

(Group 1 *versus* Group 3). No depression of the foot count ratio was produced by the i.p. inoculation of an equal amount of homogenate from an antigenically unrelated syngeneic methylcholanthrene-induced tumor (Group 2).

Table 2 summarizes the results of an experiment done to confirm the specific nature of the depression of antitumor cellular immunity produced by inoculation of tumor homogenate. While E_4 tumor-immune mice given inoculations of E_4 tumor homogenate showed the same depression of footpad reactivity demonstrated in the previous experiment (Group 3), inoculation of the same tumor homogenate into tuberculin-sensitized mice did not alter their footpad reactivity to tuberculin (Group 5). We conclude that the i.p. inoculation of E_4 tumor homogenate does not cause general immunosuppression but rather specific eclipse of the antitumor cellular immune response.

Since a homogenate of the tumor material was capable of specifically abrogating the antitumor response in tumor-immune mice, we tried to determine if the supernatant of the same material after high-speed centrifugation would also do the same. One ml of the supernatant (containing approximately 6 mg of protein) injected i.v. or i.p. did not significantly affect the level of the immune response to E_4 tumor-immune mice (data not included in Table 1 or Table 2).

This same soluble supernatant of homogenized E_4 tumor material was used to challenge E_4 tumor-immune mice to determine if it was antigenic, as determined by the footpad response. The results are given in Table 3. A slight but definite positive response was obtained when a total volume of 0.05 ml containing 3.0 mg of protein was injected into the footpads of immune mice. Differences between responses of test and control groups were small but statistically significant. An earlier experiment using 0.3 mg of the same protein also showed low but significant positivity ($p < 0.05$) (not shown). However, the response was lower than that obtained by challenge with 1×10^6 E_4 tumor cells, probably due to the presence of less antigenic material in the challenge dose. This experiment demonstrated that antigenic substances were present in the homogenized tumor material.

In an effort to isolate a fraction of the tumor homogenate capable of specifically suppressing the antitumor response of immune mice, we extracted the tumor homogenate with 3 M KCl and dialyzed the extract. Pellet 1, which was obtained by a low-speed centrifugation of the undialyzed 3 M KCl extract, did not show any suppressive activity (Group 1, Table 4). The high-speed supernatant and pellet containing

Table 3

Cellular immune response of E_4 tumor-immune mice when challenged with a high-speed supernatant of homogenized material from E_4 tumors

The challenge dose contained high-speed supernatant of material from E_4 tumors.

Group[a]	Status of mice	Foot count ratio (3 mg of protein/footpad)[b]
1	E_4 tumor-immune	1.73 ± 0.04[c]
2	Normal	1.46 ± 0.03

[a] Ten mice per group.
[b] Group 1 *versus* Group 2, $p < 0.001$.
[c] Mean ± S.E.

Table 2

Specific depression of the cellular immune response in mice given injections of tumor homogenates

Group	Status of recipient mice[a]	Treatment 24 hr earlier	Footpad challenge	Foot count ratio[b]
1	E_4 tumor-immune	None	1×10^6 E_4 cells	2.01 ± 0.12[c]
2	Normal	None	1×10^6 E_4 cells	1.42 ± 0.04
3	E_4 tumor-immune	E_4 tumor homogenate injected i.p.	1×10^6 E_4 cells	1.43 ± 0.06
4	Normal	E_4 tumor homogenate injected i.p.	1×10^6 E_4 cells	1.22 ± 0.05
5	Tuberculin-sensitive	E_4 tumor homogenate injected i.p.	10 μg *M. tuberculosis* extract	2.49 ± 0.21
6	Tuberculin-sensitive	None	10 μg *M. tuberculosis* extract	2.77 ± 0.23
7	Normal	None	10 μg *M. tuberculosis* extract	1.44 ± 0.08

[a] Ten mice per group.
[b] Group 3 *versus* 1, $p < 0.001$; Group 5 *versus* 6, $p > 0.4$, not significant.
[c] Mean ± S.E.

Table 4

Suppression of footpad reactivity in E_4 tumor-immune mice with i.p. injections of fractions from a 3 M KCl extract of the syngeneic tumor

Footpad challenge dose contained 1×10^6 E_4 tumor cells.

Group[a]	Status of recipient mice	Treatment (contents of i.p. injection 24 hr earlier)	Foot count ratio[b]
1	E_4 tumor-immune mice	Pellet 1[c]	1.78 ± 0.07[d]
2	E_4 tumor-immune mice	Pellet 2[e]	1.37 ± 0.02
3	E_4 tumor-immune mice	Supernatant 2[e]	1.47 ± 0.01
4	E_4 tumor-immune mice	None	1.98 ± 0.06
5	Normal	None	1.41 ± 0.07

[a] Nine mice per group.

[b] Group 1 *versus* 4, not significant; Group 2 *versus* 4, $p < 0.01$; Group 3 *versus* 4, $p < 0.01$.

[c] Pellet 1 obtained after low-speed centrifugation of 3 M KCl extract of E_4 tumor homogenate.

[d] Mean ± S.E.

[e] Pellet 2 and Supernatant 2 obtained after high-speed centrifugation of 3 M KCl extract of E_4 tumor homogenate (details of extraction procedure given in "Materials and Methods").

membranes, microvesicles, etc., were able to lower the cellular immune response significantly ($p < 0.01$; Groups 2 and 3 *versus* Group 4, Table 4). Although aliquots of Pellet 1 containing 10 mg of protein were injected per mouse, no suppression was seen in the immune response. The data clearly indicated that the fraction that lowered the immune response had been extracted by 3 M KCl.

***In Vitro* Suppression of Antitumor Cell-mediated Cytotoxicity Produced by the Serum of Tumor-immune Mice, but Not of Normal Mice, Inoculated with Tumor Homogenate 24 hr Earlier.** Since it appeared very likely that components from the tumor homogenate injected i.p. had disseminated through the blood stream to produce the suppression of antitumor immunity, further experiments were done to determine if the serum of mice inoculated with E_4 tumor homogenate would suppress an *in vitro* assay for cell-mediated immunity against E_4 tumor cells. The serum from both E_4 tumor-immune and normal mice given inoculations of E_4 tumor homogenate 24 hr previously were tested for suppression of a cellular cytotoxicity assay using ^{51}Cr-labeled E_4 tumor cells incubated with spleen cells from tumor-immune mice. Table 5 shows that the serum of the E_4 tumor-immune mice, but not the serum of the normal mice, suppressed antitumor cell-mediated cytotoxicity. This experiment demonstrates the specificity of the eclipse effect produced by inoculating E_4 tumor homogenate i.p.

DISCUSSION

A number of mechanisms have been postulated to explain why tumors continue to grow, despite the presence of an antitumor response on the part of the host. Many investigators have reported that blocking factors in the peripheral blood, probably consisting of antigen-antibody complexes, contribute to enhancement of tumor growth in mice (2, 7, 14). Alternately, host resistance has been reported to be lowered by increased amounts of specific antigen circulating in the peripheral blood of an animal. Specific immune paralysis in tumor host systems caused by antigen overload has been reported by Vaage (17, 18), Wepsic *et al.* (19), Deckers *et al.* (5), and Yamazaki *et al.* (20). Examples of specific desensitization due to antigen overload in systems not involving tumor cells have been described by Uhr and Pappenheimer (15), Leskowitz and Waksman (9), and Singhal and Wigzell (13).

The experiments reported in this paper demonstrate that a homogenate of tumor material injected into tumor-immune mice specifically suppressed their cellular immune response against the tumor and was not due to a generalized depression of the immune system. A high-speed supernatant and pellet of the dialyzed 3 M KCl extract of the tumor homogenate also possessed the ability to depress this cellular response. The serum of tumor-immune mice given i.p. injections of tumor homogenates also suppressed cell-mediated cytotoxicity *in vitro*. The data from the *in vitro* tests correlate well with the specific immune paralysis seen *in vivo*. It appears that this technique of electively abrogating or lowering the cellular immunity of mice gives a convenient handle to isolate the blocking factor and to study the mechanism of eclipse in this murine tumor-host system.

Table 5

In vitro suppression of antitumor cell-mediated cytotoxicity by the serum of tumor-immune mice inoculated with tumor homogenate 24 hr earlier

Group	Status of mice used as donors for serum in incubation mixture	Status of mice used as donors for spleen cells in incubation mixture	cpm of ^{51}Cr		% release
			Supernatant	Cells	
1	Tumor-immune	Tumor-immune	562 ± 39[a]	405 ± 20	58.1
2	Normal	Same	580 ± 18	411 ± 13	58.5
3	Tumor-immune mouse inoculated i.p. with tumor homogenate 24 hr earlier	Same	521 ± 23	591 ± 61	46.8
4	Normal mouse inoculated i.p. with tumor homogenate 24 hr earlier	Same	528 ± 30	389 ± 10	57.5
5	Normal	Normal	483 ± 13	446 ± 10	51.9
6	Tumor-immune	Normal	531 ± 22	460 ± 14	53.2

[a] Mean ± S.D.

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