CELLULAR IMMUNE RESPONSES TO
METHYLCHOLANThRENE-INDUCED FIBROSARCOMA
IN BALB/c MICE*

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Increasing evidence has accumulated within the last decade that neoplastic cells can
produce tumor-specific antigens within the host in which they have arisen (1, 2). For
example, the fact that preimmunization of animals with irradiated tumor cells results in
either rejection or delay of implanted tumor cell lines in the animals has been docu-
mented in a number of tumor-host systems (2, 3). Secondly, chemically induced tumors
possess tumor-specific antigens even when the tumors are morphologically identical and
arise in a single animal (4). Thirdly, specifically sensitized lymphocytes to tumor-specific
antigens are cytotoxic for the target cells (5, 6). Finally, mixed lymphocyte-tumor
reactions have been used to evaluate host reactivity to tumor cell antigens (7-9).

While the majority of the reports in experimental tumor-host systems have concen-
trated on the humoral mechanisms involved in tumor rejection or enhancement, rela-
tively little attention has been paid to the sequence of events involved in cellular immune
recognition during tumor growth. Specifically, does the cellular immune system recog-
nize tumor-specific antigens? Do immune cells release mediators of cellular reactivity?
What are the factors responsible for the loss of cellular recognition in a tumor-host
system?

In previous work, Bloom and associates (10) were able to demonstrate the presence of
migration inhibition factors in the supernates of mixtures of lymphocytes and tumor cells
which would inhibit normal guinea pig macrophages. The cytotoxic effect of immune
lymphocytes for neoplastic target cells, as well as the "enhancing" or blocking factors
which negate this effect has been well described by the Hellstroms (11, 12). However, both
the nature of the blocking factor, its actual mechanism of action, and the reasons for the
appearance on the one hand of a cytotoxic and on the other hand of a blocking factor are at
the present time unknown.

In view of the above work, it was decided to re-evaluate the host's cellular immune
response to a chemically induced fibrosarcoma in the mouse. Using both direct migration
inhibition and [14C]thymidine incorporation in DNA synthesis as measures of cellular
reactivity to tumor antigen, a sequential study of the immune response to methylcholan-
threne-induced fibrosarcoma in BALB/c mice was undertaken.

The present report indicates that there is a correlation between the degree of
cellular reactivity and the rate of tumor growth. The cellular immune response
to tumor antigens is present in the early stage of the tumor development but
after tumor growth becomes obvious, there is a progressive loss of cellular

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reactivity. Similar results were obtained in migration inhibition and blast transformation experiments in these mice. The observed loss of cellular reactivity to tumor cells appears to correlate with the appearance of blocking factors in the intraperitoneal fluid of tumor-bearing mice.

Materials and Methods

**Mouse Strains.** Female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) with methylcholanthrene-induced fibrosarcoma were obtained from Dr. Oettgen of the Sloan-Kettering Institute, New York, and both the methods of inducing the tumor, intraperitoneal passage of tumor cells, and rate of growth of this tumor have been well delineated (13). This tumor has been maintained by regular passages (625th) of $2.5 \times 10^6$ tumor cells on the 7th day of tumor growth to the peritoneal cavity of a new group of female BALB/c mice. Both the number of cells injected intraperitoneally and the day of passage of the tumor has been kept constant. In most BALB/c mice, tumor growth can be recognized grossly by the 5th day and by the 7th day the abdomen is fully distended with tumor. The animals die of this tumor within 11–15 days.

**Tissue Culture Medium.** RPMI 1640 (Associated Biomedic Systems, Inc., Buffalo, N.Y.) with 100 U/ml of penicillin and 50 µg/ml of streptomycin was used exclusively for all experiments. At the time of use, 100 µg/ml of mycostatin (E. R. Squibb and Sons, New York), and an additional 100 µg/ml of ampicillin (Wyeth Laboratories, Philadelphia, Pa.) and 10% fetal calf serum (FCS) (Flow Laboratories Inc., Rockville, Md.) were added to the basic medium. The FCS was inactivated by heating at 56°C for 1 h before use.

**Tumor Cell Lines.**

(a) Tumor cells may be obtained directly from the peritoneal cavity of tumor-bearing mice on the 7th day after injection of fresh tumor cells into the abdominal cavity of uninoculated mice. The cells were centrifuged at 1,000 rpm for 10 min; the supernate was discarded and the cell suspension washed several times in RPMI medium (Associated Biomedic Systems, Inc.).

(b) In vitro cell lines were established as follows: $0.5 \times 10^6$ cells obtained from tumor-bearing mice were inoculated into 250 ml flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) containing 75 ml of the medium described above. The medium was changed twice weekly. When the tumor cell line reached confluency (in approximately 2 wk), a new flask was started with $0.5 \times 10^6$ cells from the established culture line. These cells grow as a monolayer (often in clones) in the culture flask and adhere to the bottom of the flask. The phenomenon of contact inhibition (14) is not seen during the growth of these tumor cells. The in vitro tumor cell line has a doubling time of 8 h.

**Antigen Preparation.**

(a) Tumor cells obtained directly from the intraperitoneal cavity of BALB/c mice as well as from tissue culture lines were disrupted by sonication at 40 W for 3 min at 4°C with a microtip (Sorvall cell disruptor model W185; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). After centrifugation at 10,000 rpm for 30 min, the suspension of sonicated cells was adjusted to a concentration of 1 mg/ml (dry weight of whole membranes) and 1-ml aliquots of disrupted tumor cells were stored at -70°C until use.

(b) 4–5 million whole tumor cells were treated with 100 µg/ml of mitomycin C (Becton-Dickinson & Co., Orangeburg, N.Y.) at 37°C for $1/2$ h in 1 ml of complete RPMI medium. The trypsin blue exclusion test (15) revealed that more than 90% of the cells were still viable after mitomycin treatment but [3H]thymidine incorporation studies of the mitomycin-treated cells indicated that these cells were unable to multiply.

**Mouse Immunizations.**

(a) Cellular sensitization experiments. As described in the section on mouse strains, tumor cells were obtained from the peritoneal cavity of tumor-bearing mice. These cells were washed once and suspended in normal saline. After counting and checking the viability by the trypan blue method, a group of mice were injected with $2.5 \times 10^6$ tumor cells intraperitoneally. At appropriate intervals after tumor injection, these mice were used for the migration inhibition and blast transformation experiments.

(b) Cellular cytotoxicity experiments. For these studies, mice were immunized intraperitoneally with two doses of $4 \times 10^6$ mitomycin-treated tumor cells administered 9 days apart. Spleen cells from these mice were obtained as described below. In order to obtain the peritoneal cells for

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1 Abbreviations used in this paper: FCS, fetal calf serum; PHA, phytohemagglutinin.
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Cytotoxicity studies (16) both normal mice and mice immunized with mitomycin-treated tumor cells were injected intraperitoneally with 0.5 ml of thioglycollate (16). Unimmunized mice were sacrificed 6 days after the injection of thioglycollate. Immunized mice were injected with thioglycollate on the 3rd day after the second injection of mitomycin-treated tumor cells and sacrificed 6 days later. Spleen cells and peritoneal exudate cells were harvested from both groups of mice.

Capillary Migration Inhibition Techniques. The lymphocyte migration inhibition technique currently in use is described as follows: BALB/c mice are sacrificed by cervical dislocation at varying intervals of the time after the initiation of intraperitoneal tumor. The spleen is removed and placed in RPMI 1640 medium. Splenic lymphocytes are dissociated by gently pressing the spleen through the steel gauze nets (80 nets per square inch) with RPMI 1640 medium (no FCS). The splenic cells are centrifuged at 1,000 rpm for 10 min. Contaminating red blood cells are lysed by the addition of 5 ml of 0.83% ammonium chloride (17). The spleen cells are then washed twice in 5 ml of RPMI 1640 medium and centrifuged at 1,000 rpm for 10 min. The cells are resuspended in 10 ml of complete RPMI 1640 (10% FCS). Trypan blue exclusion tests indicate that more than 95% of the cells are viable and the suspension is adjusted to a final concentration of 4 x 10^6 cells/ml. The capillaries are then loaded with this cell suspension, sealed at one end with Seal Ease (Clay-Adams, Inc., Parsippany, N. J.), spun at 900 rpm for 10 min, and cut at the cell-fluid interface. The cut capillaries are placed in small planchettes and filled with tissue culture medium containing various concentrations of the antigen to be tested. The planchettes are sealed with cover slips and allowed to incubate for 12-18 h in a 5% CO2-air gas phase incubator at 37°C. The fans of migrating leukocytes are then projected on tracing paper and the outlines of the fans drawn on the paper. These drawings are cut out and weighed. The degree of sensitivity of the leukocytes to their specific sensitizing antigen is expressed as the percentage of inhibition. This is calculated as follows:

\[
\text{average migration of cells with antigen} \times 100 = \% \text{migration index},
\]

\[
\text{average migration of cells without antigen}
\]

where \% migration index = 100 = \% inhibition.

[^1C]Thymidine Incorporation Studies. (a) Spleen cells. Measurement of the incorporation of [^1C]thymidine (New England Nuclear, Boston, Mass.) by sensitized splenic lymphocytes in the presence of tumor antigens was carried out using a micro method modification of the standard procedure (18). BALB/c mice were sacrificed at varying intervals of time after the initiation of the intraperitoneal tumor and 2 x 10^6 ml splenic lymphocytes were suspended in RPMI 1640 with added 10% inactivated FCS. Since different lots of FCS varied considerably with respect to their ability to stimulate unsensitized spleen cells to transform, only those lots of FCS which gave minimal transformation of normal lymphocytes were used in the medium. The spleen cells were distributed at a concentration of 2 x 10^5 cells/0.1 ml in the microplate (Microtest II tissue culture plate, Falcon Plastics, Div. of BioQuest). 1 x 10^5 mitomycin-treated tumor cells in 0.1-ml vol were added to the spleen cells. Control plates of spleen cells without tumor cells were brought to the same vol of 0.2 ml with RPMI 1640 medium. Phytohemagglutinin (PHA) at a concentration of 50 \(\mu\)g/0.1 ml was used as a control. Mitomycin-treated tumor cells were also incubated alone in 0.2-ml vol. After 48 h of incubation at 37°C in CO2-air gas phase incubator, 0.01 \(\mu\)Ci of [^1C]thymidine was added to each well. After 24 h of incubation, the cells were collected on a multiple automated sample harvester [Hartzman et al. (19)], dissolved in Aquasol (New England Nuclear) and counted for 5 min on a scintillation counter (Liquid Scintillation Spectrometer, Packard model no. 3330; Packard Instrument Co., Inc., Downers Grove, Ill.).

(b) Whole blood method. Since measurement of [^1C]thymidine incorporation by sensitized lymphocytes in spleen cell suspensions necessitates sacrificing the animal, other methods were devised to measure the degree of blast transformation repetitively in a single animal. At different days after the induction of tumor, mice were bled from the left orbital sinus with heparinized small glass pipettes. 0.5 ml of blood was obtained without sacrificing the mouse and diluted with 2.5 ml of RPMI 1640 without 10% FCS and 5 x 10^{-5} M 2-mercaptoethanol. From this dilution, 0.1 ml was distributed in each well. Addition of tumor cells and the rest of the procedure was followed as described above for the spleen cells. Often, the same mice were bled twice at an interval of 4-6 days.

The blastogenic index for both methods was calculated from the [^1C]thymidine incorporation (in counts per minute) as follows:
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Cellular Cytotoxicity Studies. Tumor cells were labeled with $^{51}$Cr according to the methods of Brunner et al. (20, 21) in which $^{51}$Cr in the form of sodium chromate (Amersham/Searle Corp., Chicago, Ill.) was added at a concentration of 100 μCi/4–5 million target cells. The cells were then incubated with the label for 30 min at 37°C and then washed with Eagle’s balanced salt solution to remove excess $^{51}$Cr. For the cytotoxicity experiments, spleen cells obtained from mice immunized with mitomycin-treated tumor cells (see Mouse Immunization section) and tumor target cells were mixed in a ratio of 100:1 spleen/target cells and placed in 1 ml of complete RPMI 1640 medium. Because of the possibility that immune macrophages were needed in cytotoxicity experiments (18), other experiments were carried out in which equal numbers of spleen cells and peritoneal cells from both immunized and normal mice were added to tumor target cells at a ratio of 100 spleen/peritoneal cells to one target cell. These suspensions were also made up in 1 ml of complete RPMI 1640 medium. All suspensions were then placed in a 5% CO$_2$-air gas phase incubator at 37°C for 8 h on a continuous rotator (8 rpm). Total release of the labeled $^{51}$Cr on the target cells was determined by freeze-thaw lysis of a comparable number of target cells. Spontaneous release of $^{51}$Cr in the presence of lymphocytes obtained from nontumor-bearing BALB/c mice served as a control. After incubation, each mixture of cells was centrifuged at 800 rpm for 5 min and 0.5 ml of supernate was carefully removed and sampled for $^{51}$Cr release. The radioactivity of these samples was measured in a well-type gamma counter, Packard model no. 3002 (Packard Instrument Co., Inc.). The results were then expressed as the amount of radioactivity released from target cells in the presence of tumor immune cells as compared to the radioactivity released from target cells in nontumor immune cells. The percent of lysis was calculated as follows:

$$\frac{\left(\frac{^{51}Cr \text{ release in the presence of immune cells}}{^{51}Cr \text{ release in the presence of nonimmune cells}}\right)}{\left(\frac{\text{maximum } ^{51}Cr \text{ release from target cells}}{^{51}Cr \text{ release in the presence of nonimmune cells}}\right)} \times 100.$$  

Blocking Experiments. Intraperitoneal fluid was collected from tumor-bearing mice on the 7th day after induction of tumor. Tumor cells were separated by centrifugation at 1,000 rpm for 10 min and the supernatant fluid was collected. For each experiment, fresh fluid was obtained. Tumor-bearing mice were also bled from the orbital sinuses. The sera obtained from a number of the mice were pooled and were also used for blocking experiments.

To study the protective effect of this fluid on tumor cells in the cytotoxic experiments described above, 0.2 ml of intraperitoneal fluid or immune serum was added to the incubated mixture of immune cells and tumor cells. For control studies, normal pooled mouse serum or FCS was used.

Serological Studies. Goat antisera raised against mouse IgG, IgM, and IgA were obtained from Meloy Laboratories, Inc., Springfield, Va. Intraperitoneal fluid from mice inoculated with tumor was collected, and this fluid was studied for the presence of different classes of immunoglobulins by immunodiffusion in 1% noble agar. The antisera were lyophilized and mixed with 1 ml of intraperitoneal fluid. These mixtures were incubated for 2 h at 37°C and then overnight at 4°C. The resulting precipitate was centrifuged, and the supernate was tested for (a) residual immunoglobulin with double-diffusion studies; and (b) residual-blocking effect in the cytotoxic experiments.

Results

Migration Inhibition Experiments. A total of 77 mice were injected with $2.5 \times 10^6$ tumor cells intraperitoneally and divided into groups of 7 mice per group. Eight mice served as 0-day controls. Each group of mice was sacrificed on successive days up to the 11th day. The last group of mice was sacrificed on the 13th day after injection. Fig. 1 is a photograph of the capillary migration patterns of spleen cells obtained from tumor-bearing mice in the presence or absence of tumor antigen. As can be seen, the migration patterns of the fans
FIG. 1 A photomicrograph of the capillary migration patterns of spleen cells in the presence or absence of tumor antigens. Note the smaller fans on the right side of the photograph indicating specific inhibition of spleen cells by tumor cell antigens. × 20.

with added antigen (right side of photograph) are considerably smaller than the fan patterns in medium without antigen (left side of photograph). The experiments were always run in triplicate and only those experiments in which the fan size of a given well was similar in size to the other wells were considered valid.

The results of a number of migration inhibition studies are summarized in Fig. 2. Several observations are worthy of comment. There is a significant cellular response present on the 1st day after injection of tumor cells which reaches a peak on the 2nd day. After this time, there is a gradual loss of cellular reactivity to the tumor cells. The progressive loss of cellular recognition for tumor cells correlates with the rapid accumulation of ascites fluid and cells in the peritoneum of the mouse on the 6th or 7th day after tumor injection. After the 10th day following tumor injection, only stimulation values (migration beyond control) were seen in all mice.

Stimulation of DNA Synthesis. 32 mice were used for the [14C]thymidine incorporation studies of spleen cells. On successive days after the injection of
tumor cells 4 mice were sacrificed each day up to the 7th day and the spleen cells were obtained for DNA synthesis experiments. The last group of mice was sacrificed on the 12th day after tumor injection. Five unimmunized mice served as controls. For the whole blood studies 20 mice were injected with tumor cells. The protocol for these studies is as follows: four mice were sacrificed 24 h after tumor inoculation and successive groups of mice were sacrificed on the 3rd, 5th, and 7th day after tumor injection. The last group of mice was sacrificed on the 12th day.

The results of spleen cells blastogenesis to mitomycin-treated tumor cells are summarized in Fig. 3. As can be seen, there is a significant immune response within 24 h after the injection of tumor cells. This cellular response disappears rapidly on the 2nd day and remains so up to the time mice die of this tumor. Of interest was the fact that there is an increase in the background count of the spleen cells as the tumor progresses. The PHA response of spleen cells persists until the terminal stage of tumor growth.

The results of whole blood blastogenesis are summarized in Fig. 4. There is
rapid DNA synthesis within 24 h after induction of tumor. However, in contrast to the rapid disappearance of cellular stimulation seen in the spleen cells, the loss of cellular response to tumor antigens in whole blood is more gradual. It is not until the 7th day that there is a disappearance of the cellular response to tumor antigens and this lack of cellular reactivity remains constant until the death of the animal.

**Cellular Immune Responses to the Lower Dose of Tumor Cell Inoculation.** The rapidity with which cellular reactivity to tumor antigens was lost in
the experiments described in Fig. 2 suggested that the host's immune system was overwhelmed by the initial tumor cell inoculation. In order to test this hypothesis, the experiments described in Fig. 2 were repeated with a lower inoculating dose of tumor cells. A total of 45 mice were injected intraperitoneally with $4 \times 10^4$ number of tumor cells. Using this dose, more than 97% of BALB/c female mice developed the tumor around the 3rd wk after inoculation. These animals died of the tumor within 4–5 wk. The total number of injected mice were divided into groups of four animals. Four other normal mice served as 0-day controls. Each group of injected mice were sacrificed at different intervals as shown in Fig. 5. As noted, there is significant cellular response present on the 7th day after inoculation of tumor cells. The peak response is reached by the 14th day. After this period of time, there is a gradual loss of cellular reactivity to the tumor cells. From the 29th day onwards, these mice do not show any significant cellular response. This gradual loss of cellular reactivity corresponds to the grossly accumulating intraperitoneal tumor in these animals.

36 mice were studied for $[14C]$thymidine incorporation with spleen cells and the whole blood. Four mice served as 0-day control. Each group of four mice were sacrificed at different intervals as shown in Fig. 6. As can be seen (straight line), there is a significant cellular response by the 11th day after tumor inoculation. This response gradually disappears by the 19th day and remains negative up to the time mice die of this tumor. The dotted lines in Fig. 6 summarize the results of whole blood blastogenesis in the same animals. In general, the cellular response of the whole blood method to tumor antigens correlates well with the spleen cells response. The peak of DNA synthesis comes on the 14th day, and then there is gradual loss of reactivity up to the 19th day. After this period, the cellular response to the tumor antigen in these animals is essentially negligible up to the time of the death of the animals.

In an effort to determine the cytotoxicity effects of sensitized spleen cells with and without the addition of peritoneal cells, the following cytotoxicity experiments were performed: Spleen cells obtained from tumor immunized mice were incubated with $^{51}$Cr-labeled tumor cells. Similar experiments were carried out.
FIG. 6. The average stimulation index values of spleen cells (●—●) and peripheral whole blood (○—○) to mitomycin-treated tumor cells after lower dose inoculation of tumor cells in BALB/c mice. Peak values are noted on the 11th day and 14th day for spleen cells and whole blood cells, respectively. Values return to normal by the 19th day.

### TABLE I

**Spleen Cell-Mediated Cytotoxicity**

| No. | Average $^{51}$Cr released from each set of exp. | Cytotoxic $^{51}$Cr released by ISC |
|-----|-----------------------------------------------|-------------------------------------|
|     | TR   | SP R | N SC R | I SC R |                     |
| 1   | 825.2| 270.1| 272.2  | 297.0  | 4.8                  |
| 2   | 615.5| 136.0| 135.6  | 153.4  | 3.6                  |
| 3   | 709.0| 203.0| 205.2  | 226.1  | 4.5                  |
| 4   | 506.2| 208.0| 210.0  | 202.1  | 0                    |
| 5   | 752.0| 138.9| 142.8  | 165.5  | 4.3                  |

Abbreviations: I, immune; N, normal; R, release; SC, spleen cells; SP, spontaneous; T, total.

with peritoneal cells alone and peritoneal cells mixed with sensitized spleen cells. The results are summarized in Tables I and II.

**Cellular Cytotoxicity Studies.** Table I summarizes the results of the cytotoxicity experiments using 5 different mice. A perusal of columns two through four demonstrates that there is no significant difference in the release of $^{51}$Cr from target cells when incubated with either normal spleen cells or immune spleen cells. In addition, the spontaneous release of $^{51}$Cr from the tumor cells correlates quite well with the release of $^{51}$Cr from the normal spleen cells.

In contrast, Table II shows the results of a study of 4 mice in which the immune spleen cells were mixed with immune peritoneal cells. As can be seen, there is a significant release of $^{51}$Cr from the target cells. The percent cytotoxicity shown in the fifth column of this table clearly indicates that there is a significant cytotoxic effect of immune spleen cells for target cells when mixed with peritoneal cells.
When immune peritoneal cells alone are incubated with the $^{51}$Cr-tagged tumor cells, there is also a marked cytotoxic effect. As seen in Table III, cytotoxicity up to 88% was achieved with immune peritoneal cells. If one compares these results with the cytotoxicity values achieved with immune spleen cells and immune peritoneal cells (Table II), it is apparent that immune peritoneal cells play the major role in the cytotoxic effect on tumor cells. While not shown, spleen cells obtained from mice inoculated either intraperitoneally or subcutaneously with live tumor cells also failed to show any cytotoxic effects.

**Blocking Experiments.** The results of the blocking effect of the cell-free intraperitoneal fluid are shown in the extreme right-hand column of Table III. In the presence of the cell-free peritoneal fluid, an average of only 20.5% cytotoxicity values was obtained in the presence of the peritoneal fluid as compared to approximately 70% cytotoxic values in the absence of the fluid. Essentially identical results were obtained when pooled immune sera were used in place of the intraperitoneal fluid.

Fig. 7 shows the effect of immune cells on tumor cells when they are incubated together for 6–8 h at 37°C and were tested with trypan blue for viability. In this photograph, tumor cells are surrounded by numerous immune peritoneal cells. Trypan blue has been excluded by the immune cells and has been taken up by the tumor cells demonstrating the killing effect of the immune peritoneal cells.

**Immunoglobulin Absorption Studies.** In an effort to determine whether mouse immunoglobulins were responsible for the blocking effect on cytotoxicity experiments, absorption studies with goat antimouse immunoglobulins were carried out as described in the Materials and Methods. The results of these experiments are depicted in Fig. 8. As can be seen, all three major classes of immunoglobulins (IgG, IgM, and IgA) are present in the intraperitoneal fluid (Fig. 8 a). After absorption with specific antisera, lines of identity to the specifically absorbed class of immunoglobulins were no longer detected. These studies demonstrate that IgG, IgM, and IgA have been absorbed from the intraperitoneal fluid (Figs. 8 b, c, and d).

The results of absorbed intraperitoneal fluid studies are shown in Table IV. As can be seen, there is good cytotoxic effect of the immune peritoneal cells (91%) and intraperitoneal fluid effectively blocked the cytotoxicity (11.5%). Experiment numbers 6–8 reveal that prior absorption with specific antisera

### Table II

| No. | Average $^{51}$Cr released from each set of exp. | Cytotoxic $^{51}$Cr release % |
|-----|-------------------------------------|-----------------------------|
|     | TR        | SP R   | NSC + NPC R | ISC + IPC R |               |
| 1   | 798       | 229    | 230.9       | 438.2       | 36.7          |
| 2   | 798       | 229    | 230.9       | 359.0       | 22.8          |
| 3   | 1,102.1   | 271.2  | 269.6       | 429.2       | 19.0          |
| 4   | 1,102.1   | 271.2  | 269.6       | 451.4       | 21.8          |

Abbreviations: IPC, immune peritoneal cells; ISC, immune spleen cells; NPC, normal peritoneal cells; NSC, normal spleen cells; R, release; SP, spontaneous; T, total.
| No. | Total | Spontaneous | Normal PC | Immune PC | Immune PC with IPF | Immune PC | Immune PC with IPF |
|-----|-------|-------------|-----------|-----------|--------------------|-----------|--------------------|
| 1   | 975.2 | 144.0       | 146.5     | 858.0     | 290.0              | 85.8      | 17.5               |
| 2   | 982.6 | 320.4       | 324.0     | 834.4     | 382.3              | 77.6      | 9.3                |
| 3   | 982.6 | 320.4       | 324.0     | 903.2     | 528.0              | 88.0      | 31.3               |
| 4   | 982.6 | 320.4       | 324.0     | 805.5     | 392.0              | 73.2      | 15.6               |
| 5   | 675.1 | 103.2       | 101.3     | 594.6     | 308.2              | 85.9      | 35.9               |
| 6   | 625.0 | 175.0       | 181.0     | 368.9     | 238.9              | 43.0      | 13.7               |
| 7   | 625.0 | 175.0       | 181.0     | 412.2     | 320.6              | 52.7      | 32.7               |
| 8   | 800.3 | 283.4       | 288.0     | 560.7     | 322.6              | 53.6      | 7.5                |

Average $^{31}$Cr release values:
- $\pm$ $\leftrightarrow P < 0.01 \rightarrow$
- $\pm$ $\leftrightarrow P = \text{NS} \rightarrow$
- $\pm$ $\leftrightarrow P < 0.01 \rightarrow$
- $\pm$ $\leftrightarrow P = \text{NS} \rightarrow$
- $\pm$ $\leftrightarrow P < 0.01 \rightarrow$
- $\pm$ $\leftrightarrow P = \text{NS} \rightarrow$

Abbreviations: IPF, intraperitoneal fluid; PC, peritoneal cells.
Fig. 7. Trypan blue exclusion photomicrograph of tumor cells with immune peritoneal cells incubated for 3 h at 37°C in 5% CO₂-air gas phase. Note: Killed tumor cells with trypan blue uptake is surrounded by alive immune peritoneal cells. × 120.

Fig. 8. (a) Double diffusion studies carried out with intraperitoneal fluid and antimouse immunoglobulins. Center well contains intraperitoneal fluid. Top well contains antiserum directed against mouse IgG. Antisera to mouse IgM and IgA are placed in left and bottom wells respectively. (b, c, and d) Show loss of specific precipitation lines after absorption against specific immunoglobulin antiserum.
TABLE IV

The Effect of Immunoglobulin Absorption on Blocking of Cytotoxicity

| Average $^{51}$Cr released from each set of exp. | Cytotoxic $^{51}$Cr release % |
|-------------------------------------------------|-----------------------------|
| 1 Total release                                  | 667                         |
| 2 Spontaneous release                            | 155                         |
| 3 Normal peritoneal cells                         | 161                         |
| 4 Immune peritoneal cells                         | 620                         |
| 5 Immune peritoneal cells with intraperitoneal fluid | 214                         |
| 6 Immune peritoneal cells with IgG absorbed intraperitoneal fluid | 234 | 15.4 |
| 7 Immune peritoneal cells with IgM absorbed intraperitoneal fluid | 278 | 24.0 |
| 8 Immune peritoneal cells with IgA absorbed intraperitoneal fluid | 269 | 22.2 |

TABLE V

Cytotoxicity of Immune Peritoneal Cells Obtained from Mice Immunized with SV40-Transformed Fibroblasts and Methylcholanthrene-Induced Fibrosarcoma

| Methylcholanthrene-induced fibrosarcoma cells tagged with $^{51}$Cr | Average $^{51}$Cr released from each set of exp. | Cytotoxic $^{51}$Cr release % |
|---------------------------------------------------------------------|--------------------------------------------------|-----------------------------|
| 1 Total release                                                     | 667                                              |
| 2 Spontaneous release                                               | 155                                              |
| 3 Normal peritoneal cells                                            | 161                                              |
| 4 Immune peritoneal cells*                                           | 210                                              |
| 5 Immune peritoneal cells†                                           | 620                                              |

* Cells from mice immunized with SV40-transformed fibroblasts.
† Cells from mice immunized with methylcholanthrene-induced fibrosarcoma.

directed against the major mouse immunoglobulin classes had no effect on the blocking activity of the intraperitoneal fluid.

*Immune Response Specificity.* In an effort to determine whether the immune response of the peritoneal cells was specific for only BALB/c methylcholanthrene-induced fibrosarcoma tumor cells, the following experiments were carried out. BALB/c mice were immunized with 3 injections of mitomycin-treated SV40-transformed fibroblasts. The results of these immune peritoneal cells on methylcholanthrene-induced fibrosarcoma cells are seen in Table V. As can be seen, there is no significant release of $^{51}$Cr using SV40-immunized peritoneal cells (only 10%), as compared to the specifically immunized peritoneal cells (chromium release, 90.9%). These results strongly indicate that the cytotoxic effect of the peritoneal cells obtained from the injection of methylcholanthrene-induced tumor cells is specific for those tumor cells alone.

The question whether or not the blocking effect of the intraperitoneal fluid obtained from methylcholanthrene-induced tumor mice was specific for those
Blocking Specificity of Cell-Free Intraperitoneal Fluid on Cytotoxicity

| Methylcholanthrene-induced fibrosarcoma cells tagged with $^{31}$Cr | Average $^{31}$Cr release from each set of exp. | Cytotoxic $^{31}$Cr release |
|---------------------------------------------------------------|---------------------------------------------|-----------------------------|
| 1 Total release                                              | 667                                         | %                           |
| 2 Spontaneous release                                        | 155                                         |                            |
| 3 Normal peritoneal cells                                    | 161                                         | 1.1                         |
| 4 Immune peritoneal cells                                    | 820                                         | 90.8                        |
| 5 Immune peritoneal cells with specific intraperitoneal fluid | 214                                         | 11.5                        |
| 6 Immune peritoneal cells with nonspecific intraperitoneal fluid from radiation-induced BALB/c leukemia | 427                                         | 53.0                        |

Discussion

The examination of the cellular immune response to tumor antigens in an experimentally induced fibrosarcoma in BALB/c mice has resulted in a number of observations which are worthy of comment. First, it is clear that the use of the in vitro technique of capillary migration of mouse spleen cells is an accurate reflection of the state of cellular reactivity to tumor antigens. As noted in our studies, there was definite recognition of the tumor antigens in the first 2 days after tumor induction which rapidly disappeared culminating in stimulation values. While stimulation values were consistently seen in these mice towards the time of their death from tumor, the reasons for these values are poorly understood. The presence of a chemotactic factor associated with tumor cell antigen has not been excluded. Nor has the possibility been ruled out that the antigen-antibody complexes involving tumor antigen may be involved in this reaction.

The blastogenic studies strongly support the findings observed with the migration inhibition technique. Cellular recognition of tumor antigen was again noted early in the course of the tumor growth. The early appearance of $[^{14}$C]thymidine incorporation in mouse spleen cells (24 h) is surprising and
suggests that [\(^{14}\)C]thymidine incorporation is a more sensitive indicator of tumor antigen recognition. However, when the lower dose of tumor cells was inoculated, there was early appearance of migration inhibition factor. After the 2nd day following tumor injection no blast transformation was noted in the mouse spleen cells, yet they were capable of responding to PHA up to the late stages of tumor growth. In any event, both methods (whole blood, as well as isolated spleen cells) showed a rapid loss of ability to recognize tumor antigens. The results obtained with a lower inoculating dose strongly support the concept that cellular recognition of tumor cells is present during the early stages of tumor growth. This loss of reactivity correlates well with the gross appearance of tumor growth.

The results obtained in the cytotoxic experiments are difficult to explain but do suggest that the specific cell populations may be important in the observed cytotoxicity for tumor target cells. For example, sensitized spleen cells were not cytotoxic per se while immune macrophages exhibited marked cytotoxicity for tumor cells. These results may be open to two interpretations. The fact that cytotoxicity may be unique for macrophages suggests that the "angry" macrophage concept set forth by Macaness (22) is operating in these studies. Once immunized with tumor cells, the macrophage is the killer cell and not the lymphocyte. Alternatively, the macrophage is the effector cell in the cytotoxic experiments and has been "primed" by a sensitized lymphocyte. Experiments in which supernates from sensitized lymphocytes are mixed with normal macrophages should be performed in order to further delineate the respective roles of lymphocyte and macrophage.

Perhaps the most striking finding was the presence of "blocking" factors in the intraperitoneal fluid during growth of the tumor. This factor appears early during the course of tumor growth and effectively blocks the cytotoxic effects of macrophages for tumor target cells. The nature of this blocking factor is, at present, unknown. Absorption studies with antisera raised against the major mouse immunoglobulin classes strongly suggest that immunoglobulins per se do not play a role in this blocking effect. The same would apply to the possibility that antigen-antibody complexes are involved in the blocking effect. The concept that excess tumor antigen may be responsible for the blocking effect has not been ruled out. Preliminary experiments on Sephadex G 100 indicate that the factor is of high mol wt (>150,000) and is protein in nature (destroyed by trypsin and pepsin). It is planned to use other molecular sieves to determine the exact size of the molecule responsible for the blocking effect.

The specificity of the effector cells was demonstrated by the fact that cytotoxicity occurred only with specific immune cells and no cytotoxicity was observed with irrelevant immune peritoneal cells. In contrast, intraperitoneal fluid obtained from irrelevant tumor mice did exert a blocking effect on specific target cells. However, the effect was never as pronounced as the blocking action of specific intraperitoneal fluid. These results suggest that some of the blocking effect might be due to antigens common to both tumor cell lines, since both lines were derived from the same strain of mice.

In conclusion, the experiments described above indicate that it is possible to sequentially monitor the presence and subsequent loss of cellular reactivity to
tumor antigens in an experimentally induced tumor model in mice. Using two different in vitro parameters of cellular reactivity, it has been shown that this loss of cellular reactivity to tumor antigen correlates quite well with both the tumor growth and the appearance of a "blocking factor" in the peritoneal cavity of these mice. The nature of the blocking factor is now under investigation, but preliminary investigations suggest that major classes of mouse immunoglobulins per se are not responsible for the blocking of the cytotoxicity experiments. The reproducibility of the experiments coupled with our ability to further examine the nature of the blocking factor(s) may have important implications in our understanding of the mechanisms whereby tumor growth occurs.

Summary

Several in vitro parameters of cellular immunity were examined in BALB/c mice with an experimentally induced fibrosarcoma tumor. The results of capillary migration of spleen cells in high tumor cell dose inoculated mice show appearance of cellular immune response in the early stages of the tumor growth. As the tumor progresses, the cellular response declines and rapidly disappears, culminating in stimulation values near the time of the death of these mice.

The blastogenic studies also show early cellular recognition of tumor antigen by mouse spleen cells and whole blood (24 h). After the 2nd day following tumor injection, no blast transformation is noted. However, the results obtained with a lower inoculating tumor cell dose demonstrate an initial cellular recognition on the 7th day. This response gradually disappears by the 19th day and remains negative up to the time of the death of these mice.

This cellular immunity was confirmed by the cytotoxic experiments showing that the primary cells responsible for this cellular reactivity were the immune cells. An interesting finding was the presence of a factor(s) capable of blocking the cytotoxic effect. The nature and mechanism of this blocking factor(s) is now under investigation.

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