ANTIGEN-SPECIFIC SYNERGISM IN THE IMMUNE RESPONSE OF IRRADIATED MICE GIVEN MARROW CELLS AND PERITONEAL CAVITY CELLS OR EXTRACTS*

By JAMES C. KENNEDY,‡ M.D., PERRY E. TREADWELL,§ Ph.D., and EDWIN S. LENNOX, Ph.D.

(From the Salk Institute for Biological Studies, San Diego, California 92112, and the Department of Pathology, Queen's University, Kingston, Ontario)

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There is abundant evidence from both in vivo (1–7) and in vitro (8–15) experiments that more than one cell type contributes to the immune response of mice to sheep erythrocytes. Moreover, there seems to be agreement that the marrow contains direct precursors (1, 2, 4) of cells producing hemolysin directed against this antigen. Less clear are the roles of the cooperating cells, the number of different kinds involved, and their relative abundance in the various cell sources which have been shown to act synergistically with marrow; that is, thymus (1, 2), thoracic duct lymph (2), and spleen (5). Another source of cells known to interact with others during initiation of an immune response is the peritoneal cavity (16–20), which contains a mixture of cell types, although emphasis is usually placed on the macrophages. The results of experiments reported in this paper indicate that cells present in the marrow and in the peritoneal cavity can interact in irradiated mice to produce a synergistic immune response to foreign erythrocyte antigens; and moreover, that a synergistic immune response still occurs if the mouse peritoneal cavity cells are replaced by those from rats or by an aqueous extract made by heating such cells. The activities of the marrow cells, the peritoneal cavity cells, and the extract of the peritoneal cavity cells are antigen dependent, in the sense that cells or extracts from animals previously immunized with the test antigen produce much stronger synergistic immune responses than do cells from unimmunized animals. Prior immunization of the donor animals is often not required if sheep erythrocytes are used, since most adult mice and rats are already immune, although the degree of immunity varies greatly from one group of animals to another.

Materials and Methods

Animals.—(CA)F_1(Balb/c × A) mice were obtained from the Jackson Laboratory, Bar Harbor, Me., at about 6 wk of age and were held for 2–4 wk before use. Either all males or

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all females were used in any given experiment. Young adult Sprague-Dawley rats were obtained from a local source.

Cells. Bone marrow cells were expressed from the femurs with cold medium[1] by means of a syringe and attached needle. Cells were dispersed by gentle pipetting, freed of large clumps by passage through 250 mesh stainless steel screen, washed by centrifugation at 650 *g* for 5 min, and resuspended in medium.

Peritoneal cavity cells were obtained from mice following exsanguination under ether anesthesia. 2 ml of medium were injected into the peritoneal cavity; then the skin was peeled from the abdominal muscles and the fluid recovered by means of an 18 gauge needle on a 2 ml syringe. Care was taken not to damage the surrounding tissue. Any fluid grossly contaminated with erythrocytes or inflammatory exudate was discarded. Such care was necessary to prevent spontaneous clumping of the peritoneal cavity cells. Heparin was not used nor were the mice stimulated by previous intraperitoneal injections. Rat peritoneal cavity cells were obtained in a similar manner, the main differences being that 20 ml of medium were injected and that the peritoneal cavity was opened along the mid-line to facilitate recovery of the fluid.

Thymus lobes were removed, care being taken to cut away the fascial tags in order to avoid contamination with the nearby lymph nodes, and the tissue was disrupted by gentle aspiration with a 1 ml syringe. The cell suspension was freed of clumps by passage through a 250 mesh stainless steel screen. Cells were washed by centrifugation at 650 *g* for 5 min and then resuspended in medium.

Sheep and burro erythrocytes (SRBC and BRBC) were collected in Alsever's solution and washed three times in medium. The same sheep or burro was used as donor for all experiments.

All cell injections were via the tail vein.

Cell Counting.—Cell nuclei were counted in a Neubauer chamber after appropriate dilution in 1% acetic acid containing crystal violet. SRBC and BRBC were counted in a Coulter electronic counter (Coulter Electronics, Inc., Hialeah, Fla.).

Irradiation.—Mice were prepared as cell recipients by exposure to 900 r from two opposing 220 kvp Picker X-ray tubes (Picker Corp., White Plains, N.Y.) 160 cm apart. Each had inherent filtration of 0.25 mm Cu + 1.0 mm Al. They were operated at 200 kvp, 15 ma, and gave a combined dose rate of 55 r/min to the mice in a lucite container midway between the machines.[2]

Hemolytic Plaque-Forming Cell (PFC) Assay.—The direct plaque method of Jerne, Nordin, and Henry (22) was used. Spleens were removed from exsanguinated mice, minced in medium (1 ml per spleen) and passed several times through the orifice of a syringe to produce a single cell suspension which was then filtered through a 250 mesh screen. Various dilutions were mixed with 0.7% agar containing 1 mg/ml DEAE-dextran in Earle's salts and either BRBC or SRBC, and the mixture was then overlaid on a bottom layer of 1.4% agar in phosphate buffered saline (PBS) in petri dishes. After incubation at 37°C for 1 hr, 1.5 ml of a 1/10 dilution of guinea pig serum in medium was added and the plates incubated 30 min more at 37°C. The plates were then stained with benzidine and the plaques counted.

Assay of Hemolysin and Hemagglutinin Titers.—Twofold serial dilutions of the mouse sera were made in Levine's Tris buffer, starting with 0.6 ml of a 1:5 dilution. Hemagglutinin titers were determined by addition of one drop of 2% erythrocytes to each well and incubation for 1 hr at room temperature. The last well showing hemagglutination was recorded, and then one drop of 1:10 dilution of guinea pig serum was added to each well. Hemolysin titers were read after 30 min at 37°C with constant agitation.

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[1] In all cases, the medium was Eagle's modified by Vogt and Dulbecco (21).

[2] We are grateful to Dr. Weigle and Miss McConahey of Scripps Clinic and Research Foundation for the use of their X-ray machine.

Heated Supernatant.—The once washed-peritoneal cavity cells in medium were heated at 45°–48°C for 30 min. The cells were removed by centrifugation at 15,000 *g* for 10 min, the supernatant was retained, and the pellet was taken up in a volume to give the same cell concentration as unheated cells that were to be injected. Supernatants were correspondingly diluted.

Antisera.—Mouse anti-rat spleen sera were prepared by injecting a homogenate of rat spleen in Freund's adjuvant intraperitoneally into $(CA)F_1$ mice and bleeding them out 20 days later. Rat anti-mouse spleen sera were similarly prepared by injection of mouse spleen into two rats. Rabbit anti-mouse gammaglobulin was received from Dr. Paul Knopf who had prepared it by injection of the Fab fragments of purified mouse gammaglobulins. It was absorbed with rat serum before use in these experiments.

RESULTS

Interaction of Marrow and Peritoneal Cavity Cells to Produce a Synergistic Immune Response.—Neither marrow cells nor peritoneal cavity cells alone could restore, within a short time, the immune capacity of irradiated recipients; however, mixtures of these cells could do so. Results of experiments showing this effect are given in Table I. In these experiments, fixed numbers of marrow cells, peritoneal cavity cells, and SRBC were given in a single injection. The spleens of the recipients were removed and assayed for plaque-forming cells (PFC) 10 days later, since previous growth curve experiments had shown that at this time the synergistic immune response was well developed and the immune system of the irradiated recipient was not yet capable of responding on its own to SRBC antigens.

Preliminary studies using constant numbers of cells from one source and increasing numbers of cells from the other led us to choose 2×10^6 bone marrow cells and 1×10^6 peritoneal cavity cells as the optimal combination. Higher numbers of peritoneal cavity cells often inhibited the synergistic response with 2×10^6 marrow cells or else gave too high a background response in the absence of marrow. The responses shown in Table I are of the same magnitude as those reported by Mitchell and Miller (4) for injections of 10^6 thoracic duct cells alone or of 5×10^7 thymus cells with 10^7 bone marrow cells.

Effect of Antigen on Marrow Cells and Peritoneal Cavity Cells Transplanted into Irradiated Mice.—The number of anti-sheep hemolysin-producing cells in the spleens of normal mice begins to rise above the background levels within 24–36 hr after immunization with SRBC. However, there is a delay of approximately 4 days before the synergistic immune response becomes detectable in heavily-irradiated mice injected with a suspension containing suitable numbers of marrow and peritoneal cavity cells. Similar delays are found if small numbers of spleen cells or mixtures of marrow and thymus cells are injected instead. If this delay is caused by a requirement for transplanted cells to reestablish the necessary anatomical relationships or to differentiate or multiply before taking part in the initiation of an immune response, it might be possible to detect such a requirement by noting the effect on the immune response of injecting

the marrow and peritoneal cavity cells separately, with an appropriate time interval between injections. Moreover, by experiments of this general design it becomes possible to examine in greater detail the role of antigen in the initiation of the synergistic immune response.

In one group of experiments, peritoneal cavity cells and sheep erythrocytes were injected together into irradiated recipients. Some of these recipients received marrow cells at the same time, while others were given marrow cells after intervals of up to 4 days. Spleens were assayed for plaque-forming cells 5, 7, 9, 11, and 13 days after the initial injection. There was no indication that the immune responses of those mice which had received peritoneal cavity cells

TABLE I

Interaction of Bone Marrow and Peritoneal Cavity Cells to Produce an Immune Response to Sheep Erythrocytes in Irradiated Mice

Number of cells/mouse			Plaque-forming cells/spleen*		
			Experiment No.		
Marrow	Peritoneal	SRBC	36	38	40‡
2×10^6	1×10^6	2×10^8	477	552	655 (48–1618)
2×10^6	None	2×10^8	108	72	8 (0–20)
None	1×10^6	2×10^8	43	80	28 (12–64)
None	None	2×10^8	24	8	5 (0–8)

* From pools of five or more spleens per group 10 days after transfer of cells and antigen into irradiated recipients. Results are expressed as mean number of plaques per spleen. Plaque counts were made on 4–10 plates after suitable dilution.

‡ In this experiment, spleens were assayed individually rather than as a pool. Figures in brackets indicate range of values found within each group of five spleens.

and SRBC before being given marrow cells were any different from the responses of the mice which had been given both types of cells in a single injection. Problems with survival of irradiated mice in the absence of bone marrow made it difficult to study the effect of intervals greater than 4 days between injections when peritoneal cavity cells were given first. However, when marrow cells were given first, there was no survival problem, and the interval between injections could be extended to 10 days without danger of a spurious immune response from the regenerating immune system of the host animal. Table II summarizes the results of two such experiments.

In one experiment, heavily-irradiated mice were injected with either 2×10^6 isologous marrow cells or a mixture containing 2×10^6 marrow cells and 2×10^8 SRBC. 8 days later these same mice were given either 1×10^6 peritoneal cavity cells, 2×10^8 SRBC, or a mixture containing both (lines 5–9, Tables II). At the same time, another group of mice were irradiated and injected with 2×10^6 marrow cells, 2×10^8 SRBC, and 1×10^6 peritoneal cavity cells mixed in the various combinations indicated (lines 1–4, Table II). The same peritoneal cavity cell suspension was used for both groups of mice. Two marrow cell suspensions, each a pool of cells from 20 femurs, were used. 9 days later, spleens from each group were pooled and assayed for plaque-forming cells.

The first 4 lines of Table II show results very similar to those presented in Table I; again it is apparent that both marrow and peritoneal cavity cells are required for the synergistic immune response when cells from both sources are given as a single injection. The next 5 lines summarize that part of the experiment in which the marrow and peritoneal cavity cell injections were separated by an interval of 8 days. Several points should be noted. First, marrow cells injected without SRBC and left for 8 days in the irradiated recipients appear

TABLE II

Interaction of SRBC with Marrow and Peritoneal Cavity Cells

Days between 1st and 2nd injections	1st Injection			2nd Injection		Number* of spleens assayed	Average number plaque-forming cells per spleen
	2×10^6 Marrow cells	2×10^8 SRBC	1×10^6 Peritoneal cavity cells	1×10^6 Peritoneal cavity cells	2×10^8 SRBC		
0	+	+	+	−	−	8	797
0	+	+	−	−	−	8	28
0	−	+	+	−	−	4	22
0	−	+	−	−	−	8	6
8	+	+	−	+	+	5	1755
8	+	−	−	+	+	7	285
8	+	+	−	+	−	6	62
8	+	+	−	−	+	5	160
8	+	−	−	−	+	8	58
0	+	+‡	+	−	−	10	34
2	+	+	−	+	+	10	33
4	+	+	−	+	+	10	100
6	+	+	−	+	+	10	325
8	+	+	−	+	+	10	3325

* The first nine groups belong to one experiment; spleens were pooled and assayed 9 days after the peritoneal cavity cell injection. The last five groups were assayed 4 days after the second injection.

‡ The mice of this one group were given 4×10^8 SRBC, so that the total antigen dose is equal for all five groups of this experiment.

to be no more effective in producing a synergistic immune response with peritoneal cavity cells than are marrow cells injected at the same time as the peritoneal cavity cells and SRBC. Second, the strongest immune response occurs when SRBC are present in both the marrow and the peritoneal cavity cell injections. However, this effect of two doses of antigen is not simply a secondary response by the irradiated mice or by the transplanted marrow cells, since a second injection of SRBC in the absence of peritoneal cavity cells is relatively ineffective (line 8, Table II). Nor is the effect caused by an increase in total antigen dose, since in other experiments we have shown that a dose of

1×10^8 SRBC given with both marrow and peritoneal cavity cell injections is far more effective than is a single dose of 2×10^8 SRBC given with either the marrow or the peritoneal cavity cell injection but not with both. It appears then that the injection of SRBC into irradiated mice bearing marrow grafts does not in itself lead to a detectable immune response, but it does have a definite enhancing effect on the ability of such mice to respond to a second injection containing both SRBC and peritoneal cavity cells. The other experiment of Table II (lines 10–14) shows how this enhancing effect varies as the time interval between marrow and peritoneal cavity cell injections is varied.

Irradiated recipients were given 2×10^6 marrow cells and 2×10^8 SRBC; after intervals of either 0, 2, 4, 6, or 8 days, 1×10^6 peritoneal cavity cells and 2×10^8 more SRBC were injected. The times chosen for irradiation and marrow cell injection were such that all mice could be given peritoneal cavity cells on the same day and from the same pool; each of the five different marrow cell suspensions was made from the pooled cells of 20 femurs. Spleens were assayed for plaque-forming cells 4 days after the second injection, since we had found that when irradiated mice are given marrow and SRBC a few days before they receive a second injection containing peritoneal cavity cells and more SRBC, the growth curves of the synergistic immune response do not show the initial delay of 4 days found when both cell types are injected simultaneously but instead are very similar to those of intact mice.

As the data of the last 5 lines of Table II indicate, there is very little immune response 4 days after the simultaneous injection of marrow cells, peritoneal cavity cells and SRBC, but there is a progressively stronger response as the interval between marrow and peritoneal cavity cell injections increases. We could extend this interval up to about 10 days without detectable response by the irradiated recipient's regenerating immune system.

The experiment summarized in Table III is one of several designed to examine the specificity of the part played by antigen during initiation of the synergistic immune response.

Irradiated mice were divided into three equal groups; all received 2×10^6 marrow cells, but some were given in addition either 2×10^8 SRBC or 2×10^8 BRBC. 8 days later the mice were injected again; half of the mice in each group were given 1×10^6 peritoneal cavity cells and 2×10^8 SRBC, while the other half were given the same number of peritoneal cavity cells and BRBC. 10 spleens from each of the six subgroups were assayed for plaque-forming cells, 3, 4, 5, 7, and 9 days later, on both SRBC and BRBC.

The data of Table III show that by far the best response to SRBC occurred when SRBC were given with both the marrow and the peritoneal cavity cell injections, and that under these conditions the peak of the response was 4 days after the last injection. Omitting the SRBC from either injection or substituting BRBC for SRBC produced a much weaker response. We have already shown (Table II) that irradiated recipients given marrow cells and SRBC are "primed" so that the later injection of peritoneal cavity cells and more SRBC "triggers" a rapid and strong immune response. The data of Table

III confirm this result and indicate that the effect of the SRBC during both "priming" and "triggering" is antigen-specific.

The very weak response to BRBC under conditions which produced a strong response to SRBC was of great interest. We show below that if the marrow and peritoneal cavity cell donors are preimmunized with BRBC, the synergistic immune response to BRBC can be as strong as that to SRBC.

TABLE III

Specificity of Effect of Antigen Given with Marrow Cells or Peritoneal Cavity Cells from Nonimmunized Donors

Treatment*			Day of plaque assay‡				
Day 0	Day 8						
2×10^6 Bone marrow cells, plus	1×10^6 Peritoneal cavity cells, plus	Assayed on	11	12	14	15	17
SRBC	SRBC	SRBC	3930	10,250	2100	1130	2920
		BRBC	34	6	8	10	6
SRBC	BRBC	SRBC	159	64	320	276	89
		BRBC	12	13	66	16	88
BRBC	BRBC	SRBC	58	51	34	29	53
		BRBC	11	31	61	106	14
BRBC	SRBC	SRBC	7	52	889	920	730
		BRBC	3	12	8	6	8
None	SRBC	SRBC	24	180	196	938	780
		BRBC	4	19	10	5	4
None	BRBC	SRBC	10	54	26	18	34
		BRBC	4	1	8	4	22

* Mice which had been irradiated with 900 r were injected with bone marrow cells plus 2×10^8 sheep or burro erythrocytes as indicated; 8 days later they received peritoneal cavity cells and 2×10^8 erythrocytes as indicated.

‡ Average number of plaque-forming cells per spleen from pools of 10 spleens per group.

Effect of Prior Immunization of Cell Donors on the Synergistic Immune Response in Recipient Mice.—We had been concerned about wide variations in response from one experiment to another when SRBC were used as the antigen; in some experiments it was not possible to demonstrate any synergism between marrow and peritoneal cavity cells. The clue that this variability in response might be caused by variability in the state of immunization of the donor mice to antigen cross-reactive with SRBC came with the observation that deliberate immunization of the peritoneal cavity cell donors with SRBC greatly increased

the subsequent synergistic response with normal marrow cells. The possibility was raised that cells from mice which were not immune to any SRBC antigens would be incapable of responding at all; what we had considered to be a primary synergistic immune response might be dependent upon a previous accidental immunization with cross-reacting antigens. The use of BRBC as antigen provided a way to test this possibility experimentally. The very low numbers of plaques found when spleens of unimmunized mice were assayed on BRBC suggested that accidental immunization to this particular antigen was very uncommon, and, as shown in Table III, the use of marrow and peritoneal cavity cells from unimmunized mice produces a very weak synergistic response which provides a good base line for studying the effect of deliberate immunization with BRBC. Preimmunization of the donors of either the marrow or the peritoneal cavity cells (but not both) led to similar weak responses. However, as Table IV shows, there is strong synergism when both types of cells are obtained from preimmunized donors.

In these experiments, the marrow donors and the peritoneal cavity cell donors were immunized 21 and 30 days, respectively, prior to use as cell sources. Heavily irradiated recipient mice were injected with 2×10^6 marrow cells mixed with 2×10^8 BRBC; 8 days later, peritoneal cavity cells from either isologous mice or Sprague-Dawley rats were injected along with 2×10^8 BRBC; 4 days later, 8–10 spleens from each group were pooled and assayed.

The results shown in Table IV indicate that a strong synergistic response to BRBC is possible if both types of cells are obtained from preimmunized donors.

The ability of rat peritoneal cavity cells to replace mouse cells in the synergistic immune response made it possible to examine the origin of the plaque-forming cells and of the serum hemolysin. In vitro incubation of the plaque-forming cells with rat anti(CA)F_1 spleen antiserum and complement was effective in reducing the number of plaques while incubation with mouse anti-rat spleen antiserum was not. Details of these experiments are not given here, since the later demonstration that an aqueous extract of peritoneal cavity cells could replace such cells in the synergistic immune response with marrow makes it quite evident that the precursors of the plaque-forming cells were derived from the marrow cell suspension. In other experiments, we found that the hemolysin titer of serum from mice showing a strong synergistic immune response was reduced by incubation with rabbit anti-mouse immunoglobulin antiserum which was still capable of binding 19S mouse hemolysin after being absorbed with normal rat serum.

Nature of the Interaction between Marrow and Peritoneal Cavity Cells.—The observation that cells from different species can interact to produce a synergistic immune response made it seem likely that a soluble factor was involved rather than direct cell contact. The following experiment was designed to distinguish between these possibilities.

Recipient mice were prepared by irradiation and the injection of a mixture of 2×10^8 BRBC and 2×10^6 isologous marrow cells from mice immunized with 1×10^7 BRBC 19 days previously; 8 days later, the mice were given 2×10^8 BRBC and either thymus cells, untreated peritoneal cavity cells, heated (45°C for 30 min) peritoneal cavity cells, or supernatant from these heated cells. Both thymus and peritoneal cavity cells were obtained from mice immunized with BRBC 8 days before use, or from rats immunized 27 days previously. The results of assays 4 days after the last injection are summarized in Table V.

It can be seen that heated peritoneal cavity cells and the supernatant from these cells are effective in increasing the PFC and hemolysin response, and that the response obtained is about the same as that obtained from 50 times more thymus cells. There is one discrepancy in the Table; the PFC response of

TABLE IV

Effect of Immune Status of Donors of Marrow and Peritoneal Cavity Cells on Immune Respon to Burro RBC

1st Injection*	2nd Injection*	Average number plaque-forming cells per spleen‡
Number and source of marrow cells	Number and source of peritoneal cavity cells	
2×10^6, normal mice	5×10^5, immune mice§	18
None	5×10^5, immune mice§	30
2×10^6, immune mice‖	None	110
2×10^6, immune mice‖	5×10^5, immune mice§	865
None	2.5×10^5, immune rats¶	88
2×10^6, immune mice‖	2.5×10^5, immune rats¶	1840
2×10^6, immune mice‖	2.5×10^4, immune rats¶	1140

* All irradiated mice received 2×10^8 BRBC in both 1st and 2nd injections, with an 8 day interval between these injections.

‡ Pools of eight or more spleens assayed 4 days after the 2nd injection.

§ Cells from mice injected intravenously 30 days previously with 2×10^8 BRBC.

‖ Cells from mice injected intravenously 21 days previously with 2×10^8 BRBC.

¶ Cells from rats injected intraperitoneally 30 days previously with 2×10^8 BRBC.

immune marrow cells alone is very much higher than usual. (See Table II and Table IV for usual values). This may be due to an abnormally high response by one or more of the recipient mice whose spleens were pooled for assay. Other groups showed the usual levels of response.

The question arises whether the active factor in the supernatant from heated peritoneal cavity cell suspensions was derived from the cells or from the peritoneal fluid. That it comes from the cells was shown by experiments in which repeated washing of the cells failed to remove their ability to act synergistically with marrow cells in irradiated mice. Moreover, the peritoneal fluid itself was not active. Whether thymus cells are more effective when taken from immune donors and whether a soluble thymus factor is involved has not yet been tested. Such experiments are in progress.

TABLE V

Effect of Heated Peritoneal Cavity Cells and Extracts from Immunized Donors

1st Injection,* 2×10^6 marrow cells	2nd Injection,* as indicated	Plaque-forming cells per spleen	Log_2 hemolysin titre	Number mice per group
+	None	1140	2.98	7
+	5×10^5 mouse peritoneal	470	2.70	7
−	5×10^5 mouse peritoneal	40	ND	4
+	1×10^6 heated mouse peritoneal	3800	4.13	7
+	Supernatant from 1×10^6 heated cells above	2525	4.32	7
+	5×10^5 rat peritoneal	1010	5.17	7
−	5×10^5 rat peritoneal	20	ND	1
+	1×10^6 heated rat peritoneal	3420	5.32	7
+	Supernatant from 1×10^6 heated cells above	5080	5.98	6
+	50×10^6 mouse thymus	3800	6.48	6
+	50×10^6 rat thymus	2620	5.82	6

* All mice were given 2×10^8 burro RBC at each injection. The bone marrow cells were from mice immunized 19 days previously with 1×10^7 BRBC i.v. The mouse cells used in the 2nd injection were from peritoneal cavity or thymus of mice immunized 8 days previously; the rat cell donors were immunized 27 days previously.

TABLE VI

Antigen-Specific Effect of Supernatant from Heated Peritoneal Cavity Cells

Second injection*			Average number§ plaque-forming cells/spleen
Heated rat peritoneal cavity cell supernatant,‡ in cell equivalents injected per recipient			
From BRBC-immune rats	From SRBC-immune rats	From saline-injected rats	
5×10^5	—	—	420
—	5×10^5	—	90
—	—	5×10^5	60
2.5×10^5	—	—	315
—	2.5×10^5	—	45
—	—	2.5×10^5	82
—	—	—	60

* For the first injection, all mice were given 2×10^8 BRBC and 2×10^6 isologous marrow cells from donors immunized 20 days prior to use. For the second injection, all mice were given 2×10^8 BRBC and either heated peritoneal cavity cell supernatant from one of the three groups of rat donors, or Eagle's medium as a control.

‡ Peritoneal cavity cells which had been heated at 45°C for 30 min and centrifuged to provide supernatant were from Sprague-Dawley rats immunized with BRBC, SRBC, or saline 17 days prior to use. The cells of three rats were pooled to make each suspension.

§ Average of 6–10 spleens per group.

Antigen-Specific Effect of the Factor Extracted from Immune Peritoneal Cavity Cells.—Intact peritoneal cavity cells have an effect on the synergistic immune response which can be described as antigen-specific in the sense that the cells of donor animals immune to one particular antigen do not trigger a strong synergistic immune response to other antigens. (See Table III). Supernatants from heated peritoneal cavity cells were examined to see whether or not they also have this property.

Mice were irradiated and given a mixture of 2×10^8 BRBC and 2×10^6 marrow cells from isologous donors immunized with BRBC 20 days previously. After 8 days, the recipient mice were given 2×10^8 BRBC and aliquots of supernatant from heated peritoneal cavity cells; these cells were obtained from groups of three Sprague-Dawley rats injected intraperitoneally 17 days previously with either (*a*) 3 ml of 10% BRBC, (*b*) 3 ml of 10% SRBC, or (*c*) 3 ml of saline. Recipient spleens were pooled and assayed on BRBC 4 days later. The results are summarized in Table VI.

It can be seen that, for two different concentrations of supernatant injected, the mice which were given the supernatant of peritoneal cavity cells from rats immunized with BRBC produced an immune response against BRBC, while the other mice had only background numbers of plaques. It would appear that the active factor in the supernatant is antigen-specific. However, the factor does not appear to be immunogenic, since in other experiments it was shown that, in the absence of BRBC, the extracts cannot induce an immune response either in irradiated mice carrying BRBC-immune marrow or in intact adult mice primed several months previously with BRBC.

DISCUSSION

The experimental results outlined above lead to three major conclusions: First, cells obtained from bone marrow and cells obtained from the peritoneal cavity can interact in lethally irradiated mice to produce a synergistic immune response to foreign erythrocytes. (Tables I–IV). Second, the effectiveness of the cells taking part in the synergistic immune response is antigen-specific in the sense that the response is greatest if both cell types are obtained from specifically immune donors. (Tables III and IV, and other data presented in text). Third, a material extractable by gently heating peritoneal cavity cells from immunized animals is capable of substituting for the intact cells in a synergistic immune response with marrow cells. (Tables V and VI). This material is antigen-specific in the same sense as are the cells from which it is derived (Table VI).

Synergistic interactions of marrow have been reported previously with cells from thymus (1, 2, 14), thoracic duct lymph (2), and spleen (5). It will be of great interest to learn whether or not the mechanism of action and the cell

types involved are the same in each case. Cells capable of producing a synergistic immune response with marrow and sharing the density characteristics of thymus cells have been isolated from thoracic duct lymph (J. S. Haskill, G. F. Mitchell, and J. F. A. P. Miller, personal communication), but up to the present such cells have not been reported from the peritoneal cavity. The relative proportion of the active cells may be quite different in cell suspensions obtained from different sources; although comparable numbers of cells from the peritoneal cavity and thoracic duct lymph produce quite comparable levels of synergistic immune response with marrow cells, 50 times that number of thymus cells are required to produce a similar response (4). It is possible that the active cells of thymus form a very minor part of the total thymus population.

Cells from specifically immune donors produce a much better synergistic response than do cells from untreated animals. This was most apparent when burro erythrocytes were used (Tables III and IV), since there is little "natural" immunity to this antigen, but the effect of preimmunization of donors was also clear when sheep erythrocytes were used. However, the great variability in the synergistic immune response to sheep erythrocytes when cells from untreated donors were used strongly suggested that there was great variation in the immune status of these donor mice. A similar conclusion was reached by Nossal et al. (23). In view of this variability, it seems unfortunate that so many studies of the "primary" immune response use sheep erythrocytes as antigen; burro erythrocytes would seem preferable.

The active factor obtained from heated peritoneal cavity cells increases with prior immunization of the donors, and is antigen-specific in its effect on irradiated mice bearing marrow grafts. Since it is antigen-specific in its effect, it can not be acting merely as an adjuvant or as a nonspecific growth factor (24). Processed antigen (25, 26), specific antibody (27), or specific RNA (28, 29) would seem to be possibilities, not all equally likely. Since neither the peritoneal cavity cells nor the supernatant obtained from them appears to be capable of triggering an immune response in the absence of antigen, even in primed animals, the active factor is probably not some form of processed antigen. The reported effects of specific RNA from immunized animals include the appearance of donor antigens on the resulting antibody (28, 29). Although the hemolysin produced in irradiated mice given mouse marrow and rat peritoneal cavity cells had detectable mouse antigens, we were unable to detect any rat antigens by means of mouse anti-rat gammaglobulin antiserum. Consequently, some form of antibody is at present the most likely agent. Attempts to isolate and characterize the active factor are now in progress.

The mechanism by which prior immunization of donor animals increases the

potential of their marrow cells for synergistic activity following transplantation may be related to the mechanism by which the presence of antigen in irradiated mice bearing marrow grafts "primes" the cells so that the later injection of more antigen and peritoneal cavity cells or extract triggers a strong and rapid response (Table II). Either antigen-induced differentiation or antigen-induced proliferation may be involved. The possible involvement of the regenerating thymus or reticuloendothelial system of the irradiated hosts remains to be investigated.

SUMMARY

A synergistic immune response to foreign erythrocytes may be induced in heavily irradiated mice injected with a mixture of isologous cells obtained from marrow and from the peritoneal cavity. Under appropriate conditions, homologous or heterologous peritoneal cavity cells, heat-killed cells, or cellfree extracts made from such cells are also effective. The activity of the peritoneal cavity cells or extracts is antigen-specific, in the sense that cells or extracts obtained from animals previously immunized with the test antigen produce much stronger synergistic effects than do cells from animals immunized with some other antigen; however, the peritoneal cavity cells or extracts are not immunogenic when tested in primed animals. The marrow cells, demonstrated to contain precursors of the antibody-forming cells produced during this synergistic immune response, also show a form of antigen-specificity.

REFERENCES

1. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Immunocompetence of transferred thymus-marrow cell combinations. *J. Immunol.* **97**:828.
2. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* **59**:296.
3. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:801.
4. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.
5. Radovich, J., H. Hemingsen, and D. W. Talmage. 1968. The enhancing effect of bone marrow cells on the immune response of irradiated mice reconstituted with spleen cells from normal and immunized donors. *J. Immunol.* **100**:756.
6. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combinations. Synergism in antibody production. *Proc. Soc. Exp. Biol. Med.* **122**:1167.

7. Albright, J. F., T. F. Omer, and J. W. Deitchman. 1970. Antigen competition: antigens compete for a cell occurring with limited frequency. *Science (Washington)*. **167**:196.
8. Mosier, D. E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science (Washington)*. **158**:1573.
9. Mosier, D. E., and L. W. Coppleson. 1968. A three-cell interaction required for the induction of the primary immune response *in vitro*. *Proc. Nat. Acad. Sci. U.S.A.* **61**:542.
10. Mosier, D. E. 1969. Cell interactions in the primary immune response *in vitro*: A requirement for specific cell clusters. *J. Exp. Med.* **129**:351.
11. Raidt, D. J., R. I. Mishell, and R. W. Dutton. 1968. Cellular events in the immune response. Analysis and *in vitro* response of mouse spleen cell populations separated by differential flotation in albumin gradients. *J. Exp. Med.* **128**:681.
12. Haskill, J. S., P. Byrt, and J. Marbrook. 1970. *In vitro* and *in vivo* studies of the immune response to sheep erythrocytes, using partially purified cell preparations. *J. Exp. Med.* **131**:57.
13. Mosier, D. E., F. W. Fitch, and D. A. Rowley. 1970. Cellular deficit in thymectomized mice. *Nature (London)*. **225**:276.
14. Munro, A., and P. Hunter. 1970. *In vitro* reconstitution of the immune response of thymus-deprived mice to sheep red blood cells. *Nature (London)*. **225**:277.
15. Pierce, C. W., and B. Benacerraf. 1969. Immune response *in vitro*: independence of "activated" lymphoid cells. *Science (Washington)*. **166**:1002.
16. Feldman, M., and R. Gallily. 1967. Cell interactions in the induction of antibody formation. *Cold Spring Harbor Symp. Quant. Biol.* **32**:415.
17. Argyris, B. F. 1967. Role of macrophages in antibody production. Immune response to sheep red blood cells. *J. Immunol.* **99**:744.
18. Bussard, A. E. 1967. Primary antibody response induced *in vitro* among cells from normal animals. *Cold Spring Harbor Symp. Quant. Biol.* **32**:465.
19. Unanue, E. R., and B. A. Askonas. 1968. The immune response of mice to antigen in macrophages. *Immunology*. **15**:287.
20. Kornfeld, L., and W. W. H. Weyzen. 1968. Antibody formation by transferred peritoneal cells and spleen cells of mice. I. Transfer of cells from immunized nonirradiated donors to syngeneic recipients with and without antigen. *Immunology*. **15**:751.
21. Vogt, M., and R. Dulbecco. 1963. Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. *Proc. Nat. Acad. Sci. U.S.A.* **49**:171.
22. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. *In* Cell-Bound Antibodies. B. Amos and H. Koprowski, editors. Wistar Institute Press, Philadelphia, Pa. 109.
23. Nossal, G. J. V., K. D. Shortman, J. F. A. P. Miller, G. F. Mitchell, and J. S. Haskill. 1967. The target cell in the induction of immunity and tolerance. *Cold Spring Harbor Symp. Quant. Biol.* **32**:369.
24. Braun, W., and M. Nakano. 1967. Antibody formation: stimulation by polyadenylic and polycytidylic acids. *Science (Washington)*. **157**:819.
25. Friedman, H. P., A. B. Stavitsky, and J. M. Solomon. 1965. Induction *in vitro*

of antibodies to phage T2: antigens in the RNA extract employed. *Science (Washington)*. **149:**1106.

26. Askonas, B. A., and J. M. Rhodes. 1965. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. *Nature (London)*. **205:**470.
27. Bretscher, P. A., and M. Cohn. 1968. A minimal model for the mechanism of antibody induction and paralysis by antigen. *Nature (London)*. **220:**444.
28. Adler, F. L., M. Fishman, and S. Dray. 1966. Antibody formation initiated *in vitro*. III. Antibody formation and allotypic specificity directed by ribonucleic acid from peritoneal exudate cells. *J. Immunol.* **97:**554.
29. Bell, C., and S. Dray. 1969. Conversion of nonimmune spleen cells by ribonucleic acid of lymphoid cells from an immunized rabbit to produce γM antibody of foreign light chain allotype. *J. Immunol.* **103:**1196.