CELLS INVOLVED IN THE IMMUNE RESPONSE

VI. The Immune Response to Red Blood Cells in Irradiated Rabbits after Administration of Normal, Primed, or Immune Allogeneic Rabbit Bone Marrow Cells*

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In previous communications from this laboratory (1, 2) it was demonstrated that bone marrow cells of normal rabbits are capable of undergoing blastogenesis and mitosis upon incubation with a large number of antigens in vitro. Antibody formation by the bone marrow cells in vitro was detected by the fluorescent antibody technique (2). When a relatively large dose of an antigen was injected into a rabbit and the bone marrow removed 24 hr later, the capacity of these cells to respond to the specific antigen in vitro was lost, although the response to other non–cross-reactive antigens was intact. The injection of a lesser amount of the antigen into the donor resulted in a lengthening of the time required for the bone marrow to lose the capacity to respond to the specific antigen in vitro. Fractionation of normal bone marrow cells on a sucrose density gradient permitted the separation of a fraction high in concentration of lymphoid cells and a fraction of bone marrow containing the majority of myeloid and erythroid precursors. The former was very reactive when exposed to antigens in vitro, whereas the latter reacted only weakly in response to antigenic stimulation in vitro (2, 3). Since, of all the lymphoid tissues of the normal rabbit, only the bone marrow lymphoid cells demonstrated this capacity to react with antigens in vitro, it was concluded that the committed immunocompetent cells originate in the bone marrow of the rabbit and that they vacate the bone marrow shortly after their interaction with the particular antigen (2). We have now confirmed and extended these original observations using a cell transfer system.

It has been demonstrated, in the experiments presented below, that irradi-

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ated rabbits injected with bone marrow cells of normal rabbits are capable of forming antibodies to sheep and horse red blood cells, whereas irradiated rabbits injected with bone marrow cells obtained from rabbits injected with sheep red blood cells 24 hr prior to transfer of their cells are unable to synthesize antibodies to sheep red cells although they can to heterologous (horse) red blood cells. Data of preliminary experiments have already been presented (4).

Methods and Materials

Only outbred adult New Zealand white rabbits were used in these experiments. The antigens used were sheep red blood cells (*S*-RBC), sheep red cell stroma, horse red blood cells (*H*-RBC), and horse red cell stroma. The stroma were prepared by centrifugation of the red cells at 2000 rpm for 10 min after lysis with distilled water. The sediment, consisting of the red cell stroma, was suspended in a volume of Medium 199 (Microbiological Associates, Bethesda, Md.) equivalent to the original cell concentration.

In the experiments reported upon below, a ***primed*** rabbit is considered to be one injected with the antigen 18–24 hr prior to sacrifice, when the bone marrow has lost its capacity to respond to the antigen in vitro (2) but long before antibody can be detected in the circulation. An ***immunized*** rabbit is defined as one possessing circulating immunocompetent cells or antibody at the time of sacrifice, usually 8-10 days after antigen administration.

Normal, primed, or immune rabbits were sacrificed by the intravenous injection of Nembutal. The bone marrow from both the tibia and femur was removed and suspended in sterile normal rabbit serum (NRS) (Microbiological Associates) in sterile plastic tubes (Falcon Plastics, Los Angeles, Calif.) and shaken vigorously for 1–2 min to obtain a cell suspension, as has been previously described (1–3). The tubes were centrifuged at 600 rpm for 10 min; the fatty upper layer was decanted and the cells resuspended in Medium 199. They were washed once more in Medium 199 and resuspended in Medium 199 containing 100 units penicillin, 100 μg streptomycin, and 15% NRS/ml (Med-PS-NRS) to a cell concentration of 10^8 lymphoid cells/ml. They were kept in a 37°C incubator until they were injected intravenously into rabbits which had just been subjected to 800 R total body incidence dose irradiation using a 2000 curie cobalt-60 source with a skin source distance of 100–125 cm and an output of 60 R/min. The recipient irradiated rabbits were also injected with 10^9 *S*-RBC and were maintained in a regular animal house. The rabbits were sacrificed by the intravenous administration of Nembutal, and the spleens removed and passed through wire meshes into Med-PS-NRS to provide cell suspensions (2). The latter were analyzed for plaque-forming ability to *S*-RBC and/or *H*-RBC using the hemolytic plaque technique of Jerne and Nordin (5) with slight modifications. Agarose was used in place of agar as described in the original procedure. The spleen cells (0.1 ml), washed sheep red cells (0.1 ml of a 12% suspension), and the agarose (1 ml of a 0.5% solution) were mixed throughly in a 46°C water bath and layered into the Petri dishes which were then allowed to stand at 37°C for 2 hr. 1 ml of commercial guinea pig serum (Hyland Laboratories, Los Angeles, Calif.), diluted 10-fold, was then added. The plates were left at 37°C for another hour and the plaques were counted with the aid of a magnifying lens. The results are expressed as the number of plaque-forming cells per 10^6 splenic lymphoid cells plated. The variation in the number of plaques observed in duplicate assays was consistently less than ±10% from the mean.

Controls consisted of (*a*) normal nonirradiated rabbits injected with *S*-RBC or *H*-RBC (10^9 RBC); (*b*) normal, nonirradiated rabbits not injected with *S*-RBC or *H*-RBC; and (*c*) irradiated rabbits injected with *S*-RBC or H-RBC (10^9 RBC). The spleen cells of all these animals were tested for their plaque-forming ability to *S*-RBC and *H*-RBC.

Other rabbits were injected with *S*-RBC (10^9 cells) and sacrificed 3, 5, 6, 7, 8, 10, and 12 days later. Bone marrow, spleen, lymph node (mesenteric and popliteal), thymus, appendix, and sacculus rotundus lymphocytes were prepared as cell suspensions in Med-PS-NRS (2) and tested for plaque-forming ability to *S*-RBC and *H*-RBC. The results are expressed as the number of plaque-forming cells per 10^6 lymphoid cells tested.

Normal and primed spleen cells were also tested for their ability to undergo blastogenesis and mitosis in vitro in the presence of the specific antigen(s). The cell culture technique and method for determining the extent of radioactive ^{3}H-thymidine incorporation utilized in these experiments have been described in detail previously (2). Briefly, the cells were suspended in a concentration of 10^6/ml in Med-PS-NRS and 4 ml aliquots of the cell suspension were transferred into sterile Falcon plastic tubes. Where stated, antigen was added at the beginning of culture. The tubes were sealed and placed in a 37°C incubator for 3 days. Tritiated thymidine (2 μc, specific activity 1 c/mM) was added to the tubes 18–24 hr prior to the termination of culture, at which point the tubes were centrifuged at 2000 rpm for 10 min, washed twice with 5% trichloroacetic acid, and digested overnight with Hyamine (Packard Instruments, Downers Grove, Ill.). The cell digests were then transferred to scintillation counting vials with ethanol; 15 ml of scintillation solution was added to each vial, and the radioactive content of each vial was determined with a Packard Model 4000 liquid scintillation counter.

Antisera to *S*-RBC and *H*-RBC were prepared in rabbits by the intravenous administration of 1 ml of a 10% suspension of the red cells at weekly intervals for 3 wk. The rabbits were bled 7–10 days after the last injection, and the sera obtained after centrifugation of the clotted bloods were stored at -10°C until used.

The data presented in each table are representative of a typical experiment. Each value presented in the tables below represents the mean of duplicate or triplicate determinations obtained with the tissues of two or three different rabbits. Each experiment was repeated in its entirety in an identical fashion at least three times except where otherwise stated. In view of the consistent nature of the results obtained, not a single experiment of the more than 50 comprising this study was deleted. It must also be emphasized that the variations in the number of plaques observed with the tissues of different rabbits, treated in an identical fashion and under the same experimental conditions, were found not to exceed ± 10% of the mean value. Therefore, for the sake of simplicity of presentation, the ranges in the numbers of plaques obtained have been omitted from the tables.

EXPERIMENTAL PROCEDURES AND RESULTS

A. Plaque-Forming Ability of the Different Rabbit Lymphoid Tissues and the Optimal Conditions for the Induction of Primed Bone Marrow In Vivo and of Hemolytic Plaques In Vitro (Experiments I–IV).—This initial sequence of experiments was carried out to establish base line values for the various parameters utilized in the experimental protocols outlined below. They constitute the framework upon which the interpretations of the results of the subsequent experiments are based.

Rabbits were injected intravenously with 10^6, or 10^7, or 10^8 or 10^9 *S*-RBC and sacrificed 7 days later. Cell suspensions of the various lymphoid organs were prepared and were analyzed for plaque-forming capacity of *S*-RBC plated in constant or varying concentrations of lymphoid cells. Bone marrow suspensions were also prepared from rabbits injected with varying numbers of *S*-RBC 24 hr previously and injected into irradiated recipients. The latter were sacrificed 7 days later, and the plaque-forming capacity of the spleen cells was determined.

As can be seen in Table I, only the spleen cells of immunized rabbits were capable of forming hemolytic plaques with *S*-RBC. The maximum immune reactivity of the spleen was attained between days 6 and 8 after immunization. Very few plaques could be detected before day 3 and after day 12.

As can be seen in Table II, the administration of 10^9 *S*-RBC resulted in the

TABLE I

Plaque-Forming Capacity of Different Lymphoid Organs of Rabbits Immunized with Sheep Red Blood Cells (10^9 Cells)

Time after antigen administration	Cells of lymphoid organ plated					
	Spleen	Thymus	Bone marrow	Lymph node	Sacculus rotundus	Appendix
days	*No. of plaques/10^8 lymphoid cells*					
3	5	<1	<1	1	3	1
5	15	<1	<1	2	1	1
6	54	2	<1	2	2	2
7	73	<1	2	7	2	1
8	66	<1	1	<1	1	1
10	31	<1	2	<1	1	<1
12	8	<1	<1	1	4	<1

TABLE II

Plaque-Forming Capacity of Splenic Lymphoid Cells of Rabbits Immunized with Varying Doses of Sheep Red Cells 7 Days Prior to Sacrifice

No. of sheep red cells administered	No. of plaques/ 10^6 spleen lymphoid cells plated (day 7)
10^9	71
10^8	47
10^7	5
10^6	1

production of the maximum number of plaques by the spleen cells on the 7th postimmunization day. The maximum number of plaques was observed when 2×10^6 to 8×10^6 splenic cells were plated. Greater numbers of splenic cells (24×10^6 or 32×10^6) tended to exert an inhibitory effect on the system resulting in an almost complete inhibition of plaque formation (Table III).

As can be seen in Table IV, bone marrow cells of donor rabbits given 10^8, 10^9, or 10^{10} *S*-RBC were incapable of transferring plaque-forming capacity with respect to *S*-RBC to the irradiated, recipient rabbits, whereas this activity was possessed by bone marrow cells obtained from rabbits injected with 10^6 or 10^7 *S*-RBC 18–24 hr prior to sacrifice.

On the basis of these initial experiments it became clear that the optimal

number of hemolytic plaques would be obtained by plating 2×10^6–8×10^6 spleen cells of rabbits given 10^9 *S*-RBC 6–8 days prior to sacrifice. Furthermore, the bone marrow cells obtained from a rabbit injected intravenously with 10^9

TABLE III

Relationship between Number of Splenic Lymphoid Cells Plated and Number of Plaques Obtained from Spleen of Same Donor

No. of splenic lymphoid cells plated ($\times 10^6$)	No. of plaques/10^6 splenic lymphoid cells*
32	1
24	7
16	28
8	62
6	67
4	69
2	69

* Spleen cells obtained from rabbit immunized 7 days earlier with 10^9 *S*-RBC intravenously.

TABLE IV

Plaque-Forming Capacity of Splenic Lymphoid Cells of Irradiated Rabbits Which Had Been Injected with Allogeneic Bone Marrow from Donors Primed with Different Doses of S-RBC

No. of *S*-RBC injected into donors*	No. of plaques/10^6 splenic lymphoid cells of recipient rabbits‡ (day 7)
10^{10}	4
10^9	8
10^8	7
10^7	23
10^6	35

* Donors were given *S*-RBC intravenously 24 hr prior to sacrifice.

‡ Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 5×10^8 bone marrow cells and 1×10^9 *S*-RBC.

S-RBC 24 hr prior to sacrifice had lost the capacity to confer immunocompetence to *S*-RBC when transferred to an irradiated recipient.

B. The Transfer of Plaque-Forming Capacity with Primed Bone Marrow Cells (Experiments V–VII).—

Rabbits were injected with 10^9 *S*-RBC intravenously and sacrificed at varying intervals of time. Bone marrow cell suspensions were prepared in the manner described above and injected intravenously into irradiated recipient rabbits together with 10^9 *S*-RBC. The latter were sacrificed 7 days later, and their spleen cells were analyzed for plaque-forming capacity.

As can be seen in Table V, the bone marrow cells of rabbits primed with *S*-RBC 24 hr prior to sacrifice were unable to confer plaque-forming capacity to spleens of recipient irradiated rabbits. On the other hand, the spleens of irradiated recipients of normal, allogeneic rabbit bone marrow displayed the

TABLE V

Plaque-Forming Capacity of Splenic Lymphoid Cells of Irradiated Recipients 7 Days after Administration of Primed (S-RBC) or Normal Allogeneic Rabbit Bone Marrow Cells

Type of bone marrow transferred (0.5×10^9 lymphoid cells)	No. of sheep red cells injected into irradiated recipient	No. of plaques/10^6 splenic lymphoid cells or irradiated recipients* (day 7)
Primed: 1 day‡	10^9	4
Normal	10^9	72
None	10^9	5
Normal	None	5

* Recipients were subjected to 800 R total body irradiation prior to administration of sheep RBC or bone marrow cells.

‡ Bone marrow cells obtained from rabbit given 1×10^9 sheep red cells, intravenously 24 hr previously.

TABLE VI

Relation between the Number of Normal or Primed Bone Marrow Cells Injected and Their Capacity to Confer Plaque-Forming Ability to the Spleens of Irradiated Recipient Rabbits 7 Days Later

No. of bone marrow cells transferred to irradiated recipient	No. of plaques/10^6 splenic lymphoid cells of recipients* receiving	
	Normal bone marrow	Primed bone marrow
9×10^8	72	11
5×10^8	71	N.D.‡
2×10^8	30	N.D.
1×10^8	12	N.D.
0.5×10^8	12	N.D.

* Recipients were subjected to 800 R followed by the injection of bone marrow cells and 1×10^9 sheep red cells given intravenously.

‡ N.D., not done.

same number of plaques as were obtained with spleens of normal rabbits immunized with *S*-RBC and tested 7 days later (compare Tables I and V). Irradiated recipients given only *S*-RBC or normal bone marrow did not exhibit plaque-forming capacity (Table V).

As can be seen in Table VI, as few as 2×10^8 normal allogeneic bone marrow cells were capable of transferring plaque-forming capacity to irradiated recipients whereas as many as 9×10^8 primed allogeneic bone marrow cells were

incapable of inducing responsiveness in spleens of irradiated recipients to any degree above background.

Table VII summarizes the results obtained with bone marrow cells transferred at different intervals of time after the administration of the antigen to the donor rabbit. The maximum loss of capacity of the bone marrow to transfer plaque-forming ability is 24–48 hr after immunization with *S*-RBC. However, most of the activity appears to be lost as early as 8 hr after the *S*-RBC injection and does not reappear until 3–5 days after *S*-RBC administration.

TABLE VII

Relation between Time of Antigen Administration (Priming) and the Capacity of Primed Bone Marrow Cells to Confer Plaque-Forming Ability to Spleens of Irradiated Recipient Rabbits 7 Days Later

Interval between antigen administration* into donor and transfer of bone marrow cells to irradiated recipient	No. of plaques/10^6 splenic lymphoid cells of irradiated recipient (day 7)‡
2 hr	69§
4 "	71
8 "	12
1 day	4
2 "	5
3 "	11
5 "	92
8 "	81
10 "	84

* 1×10^9 sheep red cells given intravenously.

‡ Recipients were subjected to 800 R total body irradiation followed by the injection of 0.5×10^9 bone marrow cells and 1×10^9 sheep red cells.

§ Control values are 72 plaques for irradiated recipients given normal bone marrow and sheep RBC and 5 plaques for irradiated recipients given sheep RBC only.

The reactivity of the bone marrow transferrred after day 5 after immunization generally exceeded that of normal bone marrow allografts.

C. The Specific Loss of Antibody-Forming Capacity to Sheep Red Blood Cells by Primed Bone Marrow Cells (Experiments VIII–X).—

Normal rabbits were injected intravenously with 10^9 *S*-RBC and sacrificed 24 hr later. The bone marrow cell suspensions prepared from these rabbits were injected into irradiated recipient rabbits along with 10^9 *S*-RBC. They were then bled at intervals of time, and the serum samples were analyzed for their anti-*S*-RBC agglutinin titers by the conventional hemagglutination test. Some of the rabbits were sacrificed at day 7, and the spleen cells were tested for their capacity to undergo blastogenesis and mitosis in the presence of the antigen in vitro.

Several bone marrow donor rabbits were passively immunized with high-titered rabbit anti-*S*-RBC antiserum 24 hr prior to sacrifice. The anti-*S*-RBC titers in these donors was 1:320 at

time of sacrifice. Recipients of these bone marrow specimens were injected with 10^9 *S*-RBC and were sacrificed 7 days later, and their spleens were analyzed for plaque-forming capacity.

As can be seen in Table VIII, spleen cells of rabbits which had received normal allogeneic bone marrow cells were stimulated to undergo mitosis and blastogenesis and to incorporate tritiated thymidine when incubated with

TABLE VIII

Antigen-Induced In Vitro Incorporation of Tritiated Thymidine by Spleen Cells of Irradiated Rabbits Given Primed (S-RBC) or Normal Allogeneic Rabbit Bone Marrow Cells

Material added to cell cultures (4 × 10^6 cells)	Incorporation of tritiated thymidine by spleen cells of irradiated recipients* of	
	Normal bone marrow cells	Primed bone marrow cells‡
	cpm	*cpm*
Nil	51	96
PHA	793	1119
S-RBC stroma	3149	89
Specific incorporation§	61	0.9

* Recipients were subjected to 800 R total body irradiation and then injected with 0.5 × 10^9 bone marrow cells and 1 × 10^9 *S*-RBC. They were sacrificed 7 days later.

‡ Donor was primed by the intravenous injection of 1 × 10^9 *S*-RBC 1 day prior to transfer.

§ Ratio of ^3H uptake by spleen cells in the presence of *S*-RBC stroma to that incorporated in the absence of *S*-RBC stroma.

TABLE IX

Humoral Immune Response to Sheep Red Blood Cells (S-RBC) of Irradiated Recipients Injected with Primed (S-RBC) or Normal Allogeneic Bone Marrow Cells

Recipient* bled at day	Hemagglutination titers of recipients given	
	Normal bone marrow	Primed bone marrow
0	0	0
7	160	0
11	640	0
14	1280	20
21	2560	40
28	640	0
35	160	0
42	160	0
49	80	0
56	0	0

* Recipients were subjected to 800 R total body irradiation followed by the injection of 5 × 10^8 bone marrow cells (normal or primed) and 1 × 10^9 *S*-RBC.

S-RBC stroma in vitro, with a specific incorporation index of 61. On the other hand, spleen cells of rabbits which had received bone marrow primed to *S*-RBC were incapable of responding in the presence of *S*-RBC stroma (specific incorporation index of 0.9), although in this particular instance they were more stimulated by phytohemagglutinin (PHA) than were the spleen cells of the rabbits which had received the normal bone marrow cells, thus attesting to

TABLE X

Plaque-Forming Capacity of Splenic Lymphoid Cells of Irradiated Recipients Which Had Received Bone Marrow Cells Obtained from Donor Rabbits Passively Immunized with Antiserum to Sheep Red Blood Cells

Treatment of bone marrow donor	Anti-*S*-RBC titer of bone marrow donor at intervals of time after injection of antiserum			No. of plaques/10^6 splenic lymphoid cells of recipient rabbits
	1 hr	4 hr	24 hr	
Rabbit anti-Sheep-RBC*	320	320	160	64
None	—	—	—	67

* Rabbit was given 5 ml of antiserum (titer 32,000) intravenously at time 0 and sacrificed 24 hr later.

TABLE XI

Cross-Reactivity between Horse and Sheep Red Blood Cells—In Vivo Response in Normal Rabbits

Cells used for immunization (1 × 10^9)	Hemagglutination titer on day				No. of plaques/10^6 splenic lymphoid cells on day 7 incubated with	
	7		14			
	Anti-*H*-RBC	Anti-*S*-RBC	Anti-*H*-RBC	Anti-*S*-RBC	*H*-RBC	*S*-RBC
S-RBC	10	640	40	25,600	7	71
H-RBC	1,280	0	40,000	80	97	5

the viability of the cells in vitro (Table VIII). Further evidence supporting the inability of primed bone marrow to transfer specific antibody formation to irradiated recipients is presented in Table IX. The recipients of normal bone marrow were able to respond with an apparently normal humoral immune response to *S*-RBC, whereas sera of recipients of *S*-RBC–primed bone marrow either possessed no circulating anti–*S*-RBC antibodies or, at most, exceedingly low titers of antibody which were delayed in onset.

Passive immunization of normal donor rabbits with homologous anti–*S*-RBC antiserum did not affect the transfer of immunocompetence by their bone marrow cells to *S*-RBC in recipient rabbits (Table X).

D. The Specificity of the Immune Response to S-RBC and H-RBC of Spleen Cells of Rabbits Injected with Bone Marrow Cells from Rabbits Primed with Sheep and/or Horse Red Blood Cells (Experiments XI–XIV).—

Normal rabbits were injected intravenously with either *S*-RBC and/or *H*-RBC (10^9 cells) and bled at intervals of time. The serum samples were tested for their agglutinin titers to these two red cell preparations. Several rabbits were sacrificed on day 7, and their spleen cells were examined for their plaque-forming ability and for their capacity to incorporate tritiated thymidine in the presence of the specific antigen(s) in vitro.

As can be seen in Table XI, rabbits immunized with *S*-RBC formed antibodies essentially only to *S*-RBC, while rabbits immunized with *H*-RBC

TABLE XII

Cross-Reactivity between Horse and Sheep Red Blood Cells—In Vitro Response of Immune Rabbit Splenic Cells

Cells used for immunization	Specific incorporation* of radioactive thymidine by immune spleen cells‡ in presence of	
	H-RBC stroma	*S*-RBC stroma
S-RBC	1.2	11.0
H-RBC	13.0	0.8

* Ratio of radioactive thymidine uptake by spleen cells in presence of antigen to that taken up in the absence of the antigen.

‡ Normal rabbits were given the red cells intravenously on day 0 and sacrificed on day 7. Spleen cells (4×10^6) were incubated in vitro with either type of red cell for 3 days.

formed antibodies almost entirely directed to *H*-RBC. The plaque-forming capacity of the spleen cells of rabbits immunized with *S*-RBC produced plaques to *S*-RBC only, while spleen cells of rabbits immunized with *H*-RBC produced plaques directed to *H*-RBC only (Table XI). Similarly, spleen cells of rabbits immunized with *S*-RBC incorporated radioactive thymidine only when incubated with *S*-RBC stroma in vitro, whereas spleen cells of rabbits immunized with *H*-RBC incorporated tritiated thymidine only when incubated with *H*-RBC stroma (Table XII).

However, spleen cells of irradiated recipient rabbits injected with bone marrow cells of donors primed with *S*-RBC or *H*-RBC and immunized with both types of red cells produced plaques with respect to the heterologous antigen only, *H*-RBC or *S*-RBC, respectively (Table XIII). Spleen cells of irradiated rabbits which had received bone marrow from donors primed with both *S*-RBC and *H*-RBC were unable to form plaques to either of the red cell preparations. These data were substantiated by in vitro experiments with spleen cells of irradiated recipients. Spleen cells of irradiated rabbits which had received bone marrow primed with either *S*-RBC or *H*-RBC were capable of incorporating tritiated thymidine when incubated only with the heterologous antigen, while

TABLE XIII

Specificity of Humoral Immune Response and Plaque-Forming Capacity to Sheep and Horse Red Blood Cells of Recipients Injected with Primed (S-RBC and/or H-RBC) Allogeneic Bone Marrow Cells

Bone marrow donor primed with	Hemagglutination titer of recipient* serum at time of sacrifice		No. of plaques/10^6 of recipients* splenic lymphoid cells incubated with	
	H-RBC	*S*-RBC	*H*-RBC	*S*-RBC
S-RBC	20	0	30	4
H-RBC	0	40	5	61
S-RBC and *H*-RBC	0	0	3	4
Not primed	80	40	22	54

* Recipients were subjected to 800 R total body irradiation followed by the injection of 0.5×10^9 bone marrow cells from the specific donor and 1×10^9 *S*-RBC and *H*-RBC. All the rabbits were sacrificed on day 7.

TABLE XIV

Specificity of In Vitro Response to Sheep and Horse Red Blood Cells of Splenic Lymphoid Cells of Irradiated Recipients Injected with Primed (S-RBC or H-RBC) Allogeneic Bone Marrow 7 Days Previously

Bone marrow donor primed with	Specific incorporation* of tritiated thymidine by recipient‡ splenic lymphoid cells incubated in the presence of	
	H-RBC stroma	*S*-RBC stroma
S-RBC	5.6	0.8
H-RBC	1.2	3.7
S-RBC and *H*-RBC	1.2	0.6
Not primed	4.8	3.7

* Ratio of radioactive thymidine uptake by the spleen cells in the presence of the antigen to that taken up in the absence of the antigen.

‡ Recipients were subjected to 800 R total body irradiation followed by the injection of 0.5×10^9 bone marrow cells from the specific donor and 1×10^9 *S*-RBC and *H*-RBC. All rabbits were sacrificed on day 7. Spleen cells (4×10^6) were incubated in vitro with either type of red cell stroma for 3 days. Radioactive thymidine was added to cultures on day 2.

spleen cells of irradiated rabbits which had been injected with bone marrow of rabbits primed with both *S*-RBC and *H*-RBC were incapable of incorporating tritiated thymidine when incubated with either of the two red cell preparations in vitro (Table XIV).

DISCUSSION

The experimental protocols carried out in the present communication were designed wholly on the basis of the results presented previously by this laboratory (1–4). It had previously been demonstrated that (*a*) the bone marrow cells

of *normal* rabbits are capable of undergoing blastogenesis and mitosis, of incorporating tritiated thymidine, and of synthesizing antibody in response to the stimulus provided by a large number of antigenic materials (1, 2). (*b*) The bone marrow cells of *immunized* rabbits lose this in vitro reactivity when exposed to the specific immunizing antigen, but are as reactive as normal bone marrow cells to other, non–cross-reacting antigens (1, 2). (*c*) This in vitro reactivity of the bone marrow cells to an antigen was lost very shortly after the intravenous administration of the antigen, the eclipse or latent period not exceeding 8–24 hr when a relatively large quantity of the antigen was administered (i.e., 25 mg human serum albumin [HSA], 10 mg keyhole limpet hemocyanin [KLH]). However, it took as long as 5–10 days for the bone marrow cells to lose their reactivity to the antigen if it had been injected in only threshold amounts (i.e., 1 mg HSA) (2). (*d*) The lymphoid cells of the bone marrow, rather than other myeloid or erythroid elements of the bone marrow, were implicated as the cells reactive to the antigen stimulus in vitro (3); and (*e*) the antigen-reactive cell probably migrates out of the bone marrow after contact with the antigen in vivo (4).

Using three criteria—the hemolysis in agar (plaque) technique, the humoral immune response, and the in vitro blastogenic and mitotic response accompanied by tritiated thymidine incorporation in response to antigenic stimulation—it was unequivocally demonstrated that primed rabbit bone marrow is deficient in cells capable either of initiating or mediating the immune response to the specific antigen in an irradiated recipient. This conclusion is based on the results of approximately 50 experiments, all of which, without exception, support this interpretation. In each case, the bone marrow of rabbits injected with sheep red blood cells 18–24 hr prior to the transfer of their bone marrow to irradiated recipients failed to confer plaque-forming capacity to sheep red cells in the recipients, although the response to horse red cells, a non–cross-reacting antigen, was intact. The specificity of this loss in immunocompetence was corroborated by the failure of such recipient rabbits to form humoral antibodies to sheep red cells after antigenic stimulation and by the inability of the recipient spleen cells to undergo blastogenesis and mitosis when incubated with the specific antigen in vitro. This deletion from the bone marrow of cells capable of reacting with sheep red cells was maximum 8–48 hr after the intravenous injection of the red cells (Table VII). Thus, one may conclude that a minimum of 8 hr was required for all the antigen-reactive cells directed to sheep red cells to interact with the antigen and to vacate the bone marrow (recruitment time).

Using an entirely different system in the rat, Ford (6) arrived at a similar conclusion. Irradiated rats were injected with syngeneic thoracic duct lymphocytes either simultaneously with, or at varying times after, the injection of sheep red cells. The optimal hemolysin response was obtained in the rats given the two cell preparations simultaneously. When the delay in lymphoid cell

restoration was 12 hr or more, the latent period of the hemolysin response after the time of administration of the sheep red cells was prolonged, and the entire hemolysin response was markedly diminished. These results implied that recruitment of antigen-reactive cells in the rat occurs only over 1 to 2 days after the injection of the sheep red cells (6), which is a time interval similar to the one found in the rabbit in the present investigation.

It was demonstrated that a definite relationship exists between the number of sheep red cells used for priming the donor and the degree of loss of capacity by the bone marrow to transfer immunocompetence to the specific antigen (Table IV). These results are essentially similar to those presented previously for various protein antigens (2) and can be explained on the basis of more efficient and/or more rapid depletion from the bone marrow of the specific, precommitted cells as the number of sheep red cells injected is increased.

It was observed that the loss of capacity to transfer immunocompetence to sheep red blood cells was most conspicuous 1–2 days after the injection of this antigen into the prospective bone marrow donor. However, this ability of the bone marrow to transfer specific immunocompetence reappeared by day 4 after immunization of the donor (Table VII) and was accompanied by the demonstration of similar activity by other lymphoid organs, such as the spleen, lymph node, and appendix.[1] This finding probably reflects the appearance of a circulating pool of maturing or mature immunocompetent cells, which should now probably be classified as antibody-forming cells.

Passive immunization of the bone marrow donor with high-titered antiserum to sheep red cells did not diminish the capacity of the donor bone marrow to transfer immunocompetence to sheep red cells (Table X). The data do not conflict with those of Wigzell (7), Dixon et al. (8), Finkelstein and Uhr (9), Uhr and Moller (10), and Henry and Jerne (11), who showed that passively administered antibodies, especially 7S antibodies (11), can inhibit antibody synthesis within the same animal after an antigenic stimulus. In our experiments, only the bone marrow recipients received the antigenic stimulus.

These data, taken as a whole, strongly imply that the antigen-reactive cell in the rabbit originates in the bone marrow and that it migrates out of the marrow as soon as it interacts with the antigen. Once the antigen-reactive cells have vacated the bone marrow after interaction with the antigen, the bone marrow becomes unreactive or tolerant to this antigen and becomes deficient of the precursors of the antigen-reactive cells directed to the particular antigen. This loss is apparently permanent (2) and therefore the immunological memory which persists in the animal subsequent to the initial immune response must be a property of the cells residing in the peripheral lymphoid tissues, mainly the spleen and lymph nodes (2).

The results suggest that the bone marrow contains the different clones of

[1] Richter, M., and N. I. Abdou. Unpublished observations.

lymphoid cells which give rise to the potentially immunocompetent cells, which either transform directly into or give rise, by mitosis and differentiation, to the precommitted antigen-reactive cells. The concept of precommitment of the cell to intereact with a particular antigen encountered at some future time (2, 11, 12) accepts the tacit assumption that this cell must possess antibody-like receptor groups on its surface, as has been suggested by a number of investigators (11, 13, 14), capable of interacting with the antigen and thus initiating the inter- and/or intracellular chain of events which culminate in the humoral immune response. This interpretation of our data necessarily supports the side chain concept of Ehrlich presented almost a century ago (15) and also reaffirms the clonal selection theory of Burnet concerning the bone marrow (16). Other investigators (17–20) have also presented evidence in favor of the clonal selection theory of antibody formation, although the organ site of origin of these cells has not previously been seriously entertained.

Trentin et al. (21) have presented evidence which conflicts with the clonal selection concept. Irradiated mice which had received a minimal number of discrete colonies of splenic cells were able to respond immunologically to a number of antigens to a degree similar to that observed in nonclonal control mice. If the immunocompetent cells were indeed precommitted to the antigen and type of immune response, one would not have anticipated normal immunologic responsiveness to a number of unrelated antigens in an animal which had been repopulated after heavy irradiation with only a few stem cells and their progeny obtained from an adult donor. However, this interpretation is based on the supposition that all recipient stem cells were destroyed by the irradiation, which is hardly likely since they were subjected to only 850 R irradiation.

The role of the bone marrow in the initiation and maintenance of the immune response, and as a prime, if not only, source of immunologically-competent cells, has been discussed at length in a previous report (2). Therefore, only select references will be referred to here. Tyan and Cole (22) have presented evidence that the bone marrow serves the adult mouse as the sole source of potential immunologically competent cells. They have shown that deaths from graft-versus-host disease occurred among host mice only when they had received spleen cells from mice which had been injected with adult bone marrow. Feldman and Globerson (23) have reported that the immunological activity manifested by thymectomized mice, which had been lethally irradiated but protected with bone marrow cells and restored to immunological competence with an allogeneic thymic graft, is due to the donor marrow cells rather than to cells from the thymic graft. Results of a similar nature were obtained by Globerson (24) and Globerson and Auerbach (25) with a wholly in vitro system. The ability of the adult mouse spleen to evoke a graft-versus-host reaction in vitro was lost after sublethal irradiation but could be regenerated when such spleens

were grown in the presence of thymus tissue. However, after lethal doses of irradiation, reactivation of immune competence did not occur unless both thymus and bone marrow cells were present. They concluded that the bone marrow appeared to supply the immunocompetent cells. Goldschneider and McGregor (26) and McGregor (27) have shown that rat bone marrow can act as a source or reservoir of precursor cells which can give rise to the immuno-ogically competent lymphocyte. They demonstrated that circulating lympho-lytes capable of initiating lethal graft-versus-host reactions in the rat can develop from myeloid precursors. The most recent work of Miller and his colleagues (28–31) lends further support to the mounting evidence in favor of the bone marrow as the origin of the antibody-forming cell. They concluded from their studies that the mouse marrow cell suspensions lack adequate numbers of immunologically competent cells but contain precursor cells which mature into antibody-forming cells under the influence of the thymus. In this context, our results, which are the only ones obtained in the rabbit, concur with results obtained by all other investigators who have used mice and rats. However, our results concerning the origin of the antigen-reactive cell do not agree with these of Miller et al (28–31) who demonstrated a thymic origin for the antigen-reactive cell in the mouse. Whether the dichotomy of these results implies an altered function for some of the lymphoid organs in the ontogeny and phylogeny of the immune response in different species of animals, or whether we are dealing with bone-marrow derived cells in the thymus of the mouse or thymic-derived cells in the bone marrow of the rabbit, is unclear. This remains a problem for future experiments to elucidate.

Two questions stand out in light of the results reported in this and in the previous communications: (*a*) Do the antigen-reactive cells actually leave the bone marrow or do they become inactivated or "tolerant" to the antigen and therefore assume an incidental role in the immune response? (*b*) Do the bone marrow-derived antigen-reactive cells identified by the induction of the humoral immune response also mediate the cellular or delayed immune reaction?

Evidence has previously been presented demonstrating the capacity of normal rabbit bone marrow lymphoid cells to form antibody in vitro 5 days after their incubation with antigen, as detected by the fluorescent antibody technique (2). Other data have suggested that these antibodies are of a cytophilic type since they were not released from the cells into the supernatants of the cell cultures but were rather retained by the cells after four successive washings.[1] These findings suggest that rabbit bone marrow lymphoid cells are involved in the induction of the cell-mediated immune reactions. This interpretation of our data is supported by the recent findings of Lubaroff and Waksman (32, 33), who observed that the cell(s) mediating the "delayed" or cellular immune reaction in the rat are derived from the bone marrow. It is also not implausible to infer that this antibody-forming rabbit bone marrow

cell, which may have arisen independently from the antigen-reactive cell in the bone marrow or may be a transformed product of the antigen-reactive cell, is capable of undergoing maturation in various lymphoid organs of the irradiated recipient and to convert to an antibody-forming cell capable of synthesizing humoral antibodies. Certainly, it would appear that in the mouse and rat, the cells mediating the humoral (27–31) and cellular (32, 33) immune reactions are both derived from the bone marrow. Experiments are presently in progress to elucidate the target organ(s) for the antigen-reactive cells which leave the bone marrow in the rabbit.

SUMMARY

Irradiated rabbits given allogeneic bone marrow cells from normal adult donors responded to an injection of sheep red blood cells by forming circulating antibodies. Their spleen cells were also capable of forming many plaques using the hemolysis in gel technique, and were also capable of undergoing blastogenesis and mitosis and of incorporating tritiated thymidine upon exposure to the specific antigen in vitro. However, irradiated rabbits injected with allogeneic bone marrow obtained from rabbits injected with sheep red blood cells 24 hr prior to sacrifice (primed donors) were incapable of mounting an immune response after stimulation with sheep red cells. This loss of reactivity by the bone marrow from primed donors is specific for the antigen injected, since the immune response of the irradiated recipients to a non–cross-reacting antigen, the horse red blood cell, is unimpaired. Treatment of the bone marrow donors with high-titered specific antiserum to sheep red cells for 24 hr prior to sacrifice did not result in any diminished ability of their bone marrow cells to transfer antibody-forming capacity to sheep red blood cells. The significance of these results, with respect to the origin of the antigen-reactive and antibody-forming cells in the rabbit, is discussed.

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