CELLS INVOLVED IN THE IMMUNE RESPONSE

X. The Transfer of Antibody-Forming Capacity to Irradiated Rabbits by Antigen-Reactive Cells Isolated from Normal Allogeneic Rabbit Bone Marrow after Passage through Antigen-Sensitized Glass Bead Columns*

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Evidence has been presented demonstrating the uniqueness of the bone marrow, as compared to the other lymphoid organs, in the rabbit. It was observed that normal rabbit bone marrow cells, unlike cells of the other normal lymphoid organs, respond with blastogenesis and mitosis and incorporate tritiated thymidine following stimulation with any of a large number of antigens in vitro (1, 2). It was found that bone marrow cells obtained from an immunized rabbit sacrificed 8–24 hr following immunization ("primed" bone marrow) lose the capacity to respond to the specific antigen in vitro, although their response to other, non–cross-reacting antigens is unimpaired (2). The cell (or cells) in the normal bone marrow exhibiting this activity was identified as a lymphocyte or lymphoid cell following fractionation of the bone marrow in a sucrose density gradient (3). In a subsequent investigation (4, 5), these findings were further confirmed using a different technique, the hemolysis in gel (plaque) method of Jerne et al. (6, 7). It was observed that spleen cells of irradiated rabbits (800 R total body irradiation) given normal allogeneic bone marrow cells and immunized with sheep erythrocytes were capable of producing many hemolytic plaques in vitro. Cells of the other lymphoid organs of the recipient did not possess this activity (5). However, spleen cells of irradiated rabbits given "primed" allogeneic bone marrow cells and immunized with sheep erythrocytes were incapable of producing plaques in vitro (5). It was further demonstrated, utilizing the technique of inhibition of plaque formation with antiallotype antiserum, that the antibody-producing or plaque-forming cell in the irradiated recipient rabbit is irradiation-resistant and is of host, not donor, origin (8). This cell is therefore not derived from any of the allogeneic bone marrow cells transferred.

Thus, it appears that the immunocompetent cell in the transferred marrow capable of conferring antibody-forming capacity to an irradiated host is the

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antigen-reactive cell, and that normal bone marrow possesses no antibody-forming cells. If such is the case, it should be possible to demonstrate a direct interaction between the antigen and the antigen-reactive cell in vitro. Such an interaction occurs, as is shown below using specific antigen–sensitized glass bead columns. The passage of normal bone marrow cells through the column resulted in retention by the column of the specific antigen–reactive cells, which

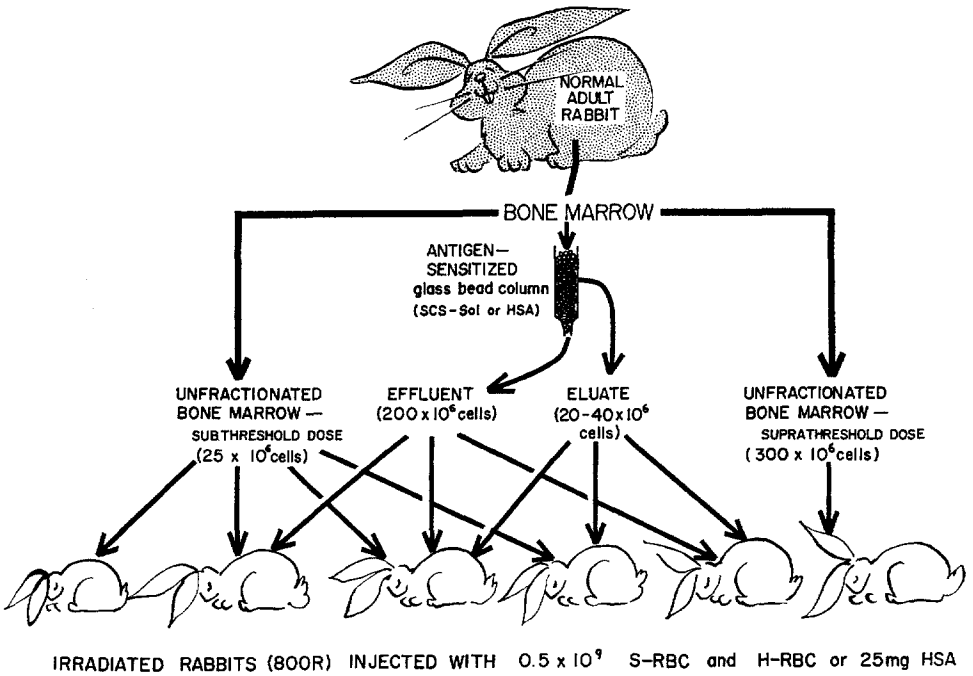

FIG. 1. Protocol for the demonstration of the specific interaction of antigen with the antigen-reactive cell in vitro.

could be eluted from the column and be shown to possess immunocompetence directed only to the specific antigen used to sensitize the glass beads.

Methods and Materials

Adult New Zealand white rabbits were used throughout this study. The antigens used were human serum albumin (HSA) (Hyland Laboratories, Los Angeles, Calif., sheep red blood cells (*S*-RBC), and horse red blood cells (H-RBC). The red cells were obtained as sterile cell suspensions in Alsever's solution. Red cell stroma were prepared by lysis of the red cells in sterile distilled water, and the stroma were then sonicated using a Fisher Ultrasonic probe (16,000 cycle/sec for 1 min). This latter material is referred to as the sheep cell sonicate (SCS) or the horse cell sonicate (HCS) preparation.

The protocol of the experimental procedures is diagrammatically presented in Fig. 1. Normal rabbit bone marrow was obtained as described previously (2). The donor rabbit was sacrificed

by the intravenous administration of Nembutal (50 mg/kg body weight). The femur and tibia were split open with a bone cutter, and the bone marrow was flushed into sterile plastic tubes (Falcon Plastics, Los Angeles, Calif.) containing 5 ml sterile normal rabbit serum (NRS). The tubes were capped, shaken for 1–2 min, and centrifuged at 600 rpm for 5 min. The fatty supernatants were discarded, and the cells were suspended in Medium 199 (Med-199) (Microbiological Associates, Bethesda, Md.) and centrifuged a second time. The cells were resuspended to a concentration of 10^8 cells/ml in Med-199 containing 15% NRS. These cells were then passed through a column of antigen-sensitized glass beads prepared according to Wigzell and Andersson (9). A column 20 cm long and 1.5 cm I.D. was used. The glass beads (Superbrite glass beads, type 100-5005, obtained from the Minnesota Mining and Manufacturing Co., St. Paul, Minn.) were boiled in nitric acid for 15 min, after which they were washed with phosphate-buffered saline (pH 7.0) (PBS) until the pH of the wash solution was 7.0. The antigen, HCS or SCS processed from 25 ml packed red cells (or 250 mg HSA), was added to 25 ml glass beads in 25 ml PBS, incubated at 45°C for 1 hr, and then left overnight at 4°C in the presence of 10% NRS. The antigen-sensitized beads were then poured into the glass column and washed with approximately 10 volumes PBS. As will be seen below, no antigen could be detected in the final effluent wash using the technique of inhibition of specific agglutination of antigen-sensitized red blood cells (10). The normal bone marrow cells (300–500 $\times$ 10^6 cells) in 3–5 ml of Med-199 were then applied to the head of the glass bead column and passed through the column with Med-199 as eluting fluid. When cells could no longer be recovered in the effluent, the glass beads were placed in a sterile 250 ml flask and vigorously shaken for 5 min. The cells in the supernatant which were eluted from the antigen-sensitized glass beads (eluate) were centrifuged and washed with Med-199. The cells in both the effluent and eluate were suspended in Med-199 and injected into allogeneic adult rabbits which had just been subjected to 800 R total body irradiation from a ^{60}Co source. Some rabbits were injected with either the effluent or eluate fraction; others received a mixture of effluent and eluate cells, and the remaining animals were injected with varying numbers of the unfractionated bone marrow cell suspension (Fig. 1). All the rabbits were injected intravenously with 0.5–1 $\times$ 10^9 *S*-RBC or *H*-RBC. Since only a small number of eluate cells were injected into the irradiated recipients, a subthreshold dose of the original unfractionated bone marrow cell suspension was also administered. All animals were sacrificed 7 days later with intravenous Nembutal (50 mg/kg body weight), and the spleens were excised and blood was obtained via intracardiac puncture. The spleen was cut into fragments which were pressed through 50 mesh wire gauze into cold Med-199 to obtain a cell suspension. These cells were analyzed for their direct plaque-forming capacity by the hemolysis in gel technique of Jerne et al. (6, 7).

Other irradiated rabbits were given bone marrow cells fractionated on the HSA-sensitized glass bead column (Fig. 1) and 25 mg HSA intravenously. The rabbits were then bled at intervals of time and the sera were tested for their antibody content by the passive hemagglutination technique (10). Hemagglutinin titers under 10 are considered to be negative in all of the tables presented.

Smears of the unfractionated bone marrow, effluent cells, and eluate cells were prepared on cleaned glass slides and stained with hemotoxylin and eosin or Giemsa stains. They were analyzed for their morphological and staining characteristics under the microscope.

The viability of the various cell fractions was determined by the dye exclusion test, using 2% trypan blue. A drop of the dye was added to 1 ml of the cell suspension, and the latter was then analyzed in a hemocytometer. The viability of the cells, on the basis of 200 cells counted, was recorded as a percentage of dead cells.

RESULTS

A. Establishment of Optimal Conditions for the Fractionation of Normal Bone Marrow Cells on Glass Bead Columns and Transfer of Immunocompetence with

Bone Marrow Cells.—As can be seen in Table I, the total number of cells recovered following passage of normal bone marrow cells through either an antigen-sensitized or an unsensitized glass bead column was in the range of 40–55% of the original cell preparation. This range of recovery, which was obtained in a total of 20 experiments, represents the number of cells recovered in both the effluent and eluate. The vast majority of the cells recovered constituted the

TABLE I

Extent of Recovery of Normal Rabbit Bone Marrow Cells Fractionated by Passage through an Antigen-Sensitized Glass Bead Column

Antigen used for sensitizing glass beads	Cells added to column		Cells obtained in effluent*		Cells eluted from glass beads‡	
	No.	Per cent of original	No.	Per cent of original	No.	Per cent of original
HSA	5×10^8	100	2.0×10^8	40	7.5×10^6	1.5
SCS	5×10^8	100	1.5×10^8	30	45.0×10^6	9.0
None	5×10^8	100	2.5×10^8	50	27.0×10^6	5.4

* Cells which passed through the column.

‡ Cells retained by the glass beads and recovered by shaking of the beads.

TABLE II

Viability of the Rabbit Bone Marrow Cells Recovered after Passage through an Antigen-Sensitized Glass Bead Column

Antigen used for coating glass beads	Viability of cells (per cent dead)*		
	Unfractionated bone marrow	Effluent fraction‡	Eluate fraction§
HSA	11	11	16
SCS	8	9	20
None	12	12	16

* Cells stained with trypan blue.

‡ Cells which passed through the column.

§ Cells retained by the antigen-sensitized glass beads and eluted by shaking.

effluent fraction. The cells which were subsequently eluted from the glass bead column, by shaking the beads in Med-199 for 5 min, constituted only 1–10% of the original cell population, with a mean value of 3–5%. It should be noted that cells could be eluted from glass beads which had not been originally sensitized with antigen (Table I).

Insofar as the viability of the fractionated cells is concerned, the effluent cells were as viable as the original unfractionated bone marrow cell suspension (Table II). The percentage of dead cells in the eluate fraction was somewhat higher, however, probably reflecting the damage incurred during vigorous shaking of the glass beads during the elution procedure (Table II).

In order to establish base line values for the following experiments, the minimum number of normal bone marrow cells required to be transferred to an irradiated host in order to confer plaque-forming capacity to the spleen cells of the recipient was determined. The administration of 5×10^8 cells has been found, in previous studies (5), to confer maximum activity. Threshold activity was obtained following the transfer of $0.5–1.0 \times 10^8$ bone marrow cells, whereas a smaller number of bone marrow cells could not confer plaque-forming activity to an irradiated recipient rabbit (Table III).

B. Isolation of Antigen-Reactive Cells Directed to HSA by Passage of Cells through an HSA-Sensitized Glass Bead Column—Passive Transfer of Specific Immunocompetence with These Cells.—Normal rabbit bone marrow cells were

TABLE III

Relationship between the Capacity of the Spleen Cells in an Irradiated Rabbit to Produce Hemolytic Plaques and the Number of Normal Bone Marrow Cells Injected along with the Antigen (S-RBC)

No. of normal rabbit bone marrow cells transferred to irradiated host* (plus 10^9 *S*-RBC)	No. of hemolytic plaques per 10^6 splenic cells of recipients on day 7
5.00×10^8	68
2.00×10^8	30
1.00×10^8	12
0.50×10^8	12
0.25×10^8	1

* Normal rabbits were subjected to 800 R total body irradiation and then given allogeneic bone marrow and 1×10^9 *S*-RBC.

fractionated by passage through an unsensitized glass bead column, yielding effluent and eluate fractions. The latter cells were injected into irradiated recipient rabbits which were also immunized with 25 mg HSA. Only recipients of effluent cells formed circulating antibodies to HSA. At no time were antibodies found in the circulation of recipients of eluate cells (Table IV).

When the cells were fractionated on an HSA-sensitized glass bead column, results of an opposite nature were obtained. In this case, only irradiated recipients of eluate cells responded with an immune response to HSA, whereas irradiated rabbits injected with effluent cells or effluent cells and a subthreshold dose of unfractionated bone marrow failed to respond upon immunization with HSA (Table V). These experiments were repeated in an identical fashion four times, with essentially identical results.

The effect of the presence of free or excess antigen in the glass bead column on the subsequent fractionation of the bone marrow cells is presented in Table VI. In this case, all the immunocompetent activity, with respect to HSA, was localized to the effluent fractions. Recipients of eluate fractions did not respond to immunization with HSA.

C. Transfer of Immunocompetence with Respect to Red Cells to Irradiated Rabbits with Fractions of Bone Marrow Obtained by Passage of Bone Marrow Cells through Red Cell–Sensitized Glass Bead Columns.—Initial experiments were aimed at demonstrating the lack of affinity of the antigen-reactive cells for

TABLE IV

Immune Response to HSA in Irradiated Rabbits Given Fractions of Normal Bone Marrow Passed through an Unsensitized Glass Bead Column

No. of bone marrow cells transferred in each fraction	Hemagglutinin titers in irradiated recipients subsequent to transfer of bone marrow*				
	Day 8	Day 12	Day 16	Day 25	Day 32
Eluate (2.5×10^6)	0‡	0	0	0	0
Effluent (3×10^8)	10	80	160	40	0
Eluate (2.5×10^6) + effluent (3×10^8)	0	160	160	80	0

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 25 mg HSA and the specified fraction of bone marrow.

‡ Titers less than 10 are considered negative.

TABLE V

Immune Response to HSA in Irradiated Rabbits Given Different Fractions of Bone Marrow Obtained by Passage of Cells through an HSA-Sensitized Glass Bead Column

No. of bone marrow cells transferred in each fraction	Hemagglutinin titers in irradiated recipients subsequent to transfer of bone marrow*					
	Day 8	Day 12	Day 16	Day 24‡	Day 30	Day 36
Unfractionated bone marrow (2.5×10^8)	0§	80	80	20	160	40
Effluent (1×10^8) + unfractionated bone marrow (2×10^7)	0	0	0	0	0	40
Eluate (4×10^6) + unfractionated bone marrow (2×10^7)	10	40	80	20	320	160
Effluent (1×10^8)	0	0	0	0	10	40
Eluate (4×10^6)	20	40	40	0	80	160
Unfractionated bone marrow (2×10^7)	0	0	0	0	0	0

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 25 mg HSA.

‡ Each rabbit received 10 mg HSA intravenously to induce a secondary immune response.

§ Titers less than 10 are considered negative.

unsensitized glass beads. Normal rabbit bone marrow cells were passed through an unsensitized glass bead column, and the effluent and eluate cell fractions obtained were administered to irradiated recipients along with the red cells. Only the spleen cells of recipients given either unfractionated bone marrow or the effluent cells of bone marrow were capable of producing plaques in vitro (Table VII). None of the recipients of the eluate fraction possessed this activity.

TABLE VI

Immune Response to HSA in Irradiated Rabbits Given Different Fractions of Bone Marrow Obtained by Passage of Cells through an HSA-Sensitized Glass Bead Column: Effect of the Presence of Unbound HSA in the Columns*

No. of bone marrow cells transferred in each fraction	Hemagglutinin titers in irradiated recipients subsequent to transfer of bone marrow‡					
	Day 8	Day 12	Day 16	Day 24§	Day 30	Day 36
Unfractionated bone marrow (1.5×10^8)	0‖	80	20	0	80	40
Effluent (0.75×10^8) + unfractionated bone marrow (2×10^7)	0	10	20	0	160	40
Eluate (2×10^6) + unfractionated bone marrow (2×10^7)	0	0	0	0	0	10
Effluent (0.75×10^8)	0	20	10	0	80	40
Eluate (2×10^6)	0	0	0	0	0	0
Unfractionated bone marrow (2×10^7)	0	0	0	0	0	0

* Last wash of the glass bead column prior to passage of the bone marrow cells contained 1μg HSA/ml of the wash.

‡ Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 25 mg HSA.

§ Each rabbit received 10 mg HSA intravenously to induce a secondary immune response.

‖ Titers less than 10 are considered negative.

TABLE VII

Plaque-Forming Capacity of Spleen Cells of Irradiated Rabbits Given Different Fractions of Normal Bone marrow Cells Obtained by Passage of Cells through an Unsensitized Glass Bead Column

No. of cells transferred to irradiated recipient in each fraction*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with sheep red cells and complement
Unfractionated bone marrow (4×10^8)	68
Eluate (13×10^6) + unfractionated bone marrow (25×10^6)	<1
Effluent (10^8) + unfractionated bone marrow (25×10^6)	33
Eluate (13×10^6), effluent (10^8), and unfractionated bone marrow (25×10^6)	31

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 10^9 *S*-RBC.

Similarly, passage of cells through an HSA-sensitized glass bead column resulted in the localization of all the antigen-reactive cells directed to sheep red cells in the effluent fraction. Here as well, the eluate fraction could not transfer immunocompetence directed toward the sheep red cell (Table VIII).

When the bone marrow cells were fractionated using a glass bead column sensitized with the solubilized sheep red cell stroma preparation (SCS), all the antigen-reactive cells directed to sheep red cells were retained in the column,

since the effluent cells possessed no capacity to transfer plaque-forming ability to sheep red cells in irradiated recipients (Table IX). The cells eluted from the SCS-sensitized glass bead column possessed all the antigen-reactive cells directed toward sheep red cells, since they could confer considerable plaque-forming capacity on the spleens of irradiated recipients (Table IX).

TABLE VIII

Plaque-Forming Capacity of Spleen Cells of Irradiated Rabbits Given Different Fractions of Normal Bone Marrow Cells Obtained by Passage of Cells through an HSA-Sensitized Glass Bead Column

No. of cells transferred to irradiated recipient in each fraction*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with sheep red cells and complement
Unfractionated bone marrow (25×10^6)	1
Eluate (17.5×10^6) + unfractionated bone marrow (25×10^6)	5
Effluent (260×10^6) + unfractionated bone marrow (25×10^6)	68
Effluent (260×10^6)	45

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 10^9 *S*-RBC.

TABLE IX

Plaque-Forming Capacity of Spleen Cells of Irradiated Rabbits Given Different Fractions of Normal Bone Marrow Cells Obtained by Passage of the Cells through a Sheep Red Cell–Sensitized Glass Bead Column

No. of cells transferred to irradiated recipient in each fraction*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with sheep red cells and complement
Unfractionated bone marrow (5×10^8)	87
Eluate (22×10^6) + unfractionated bone marrow (25×10^6)	49
Effluent (75×10^6) + unfractionated bone marrow (25×10^6)	1
Eluate (22×10^6), effluent (75×10^6), and unfractionated bone marrow (25×10^6)	53

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injection of 10^9 *S*-RBC.

The antigenic specificity of the cells retained by the antigen-sensitized glass bead columns was further established by fractionating bone marrow cells on two columns sensitized with soluble stromal preparations of two non–cross-reactive red cell species—sheep (SCS) and horse (HCS) erythrocytes. In each case (Tables X and XI), the sensitized glass bead column retained the antigen-reactive cells directed only to the antigen used to sensitize the column.

The passage of normal bone marrow cells through an HCS-sensitized column resulted in the retention on the column of horse, but not sheep, red cell–reactive

cells (Table XII). However, passage of the effluent (sheep red cell–reactive) cells, recovered from the HCS column, through an SCS-sensitized column

TABLE X

Plaque-Forming Capacity, with Respect to Sheep and Horse Red Cells, of Spleen Cells of Irradiated Rabbits Given Bone Marrow Cells Fractionated on a Sheep Red Cell–Sensitized Glass Bead Column

No. of cells injected into irradiated recipient in each fraction*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with	
	H-RBC	*S*-RBC
Unfractionated bone marrow (2.8×10^8)	45	63
Unfractionated bone marrow (25×10^6)	6	3
Eluate (10×10^6)	4	37
Eluate (10×10^6) + unfractionated bone marrow (25×10^6)	2	48
Effluent (1.5×10^8)	40	5
Effluent (10×10^6)	4	3
Effluent (1.5×10^8) + unfractionated bone marrow (25×10^6)	49	9

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injections of 10^9 *S*-RBC and 10^9 *H*-RBC.

TABLE XI

Plaque-Forming Capacity, with Respect to Sheep and Horse Red Cells, of Spleen Cells of Irradiated Rabbits Given Bone Marrow Cells Fractionated on a Horse Red Cell–Sensitized Glass Bead Column

No. of cells injected into irradiated recipient in each fraction*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with	
	H-RBC	*S-RBC*
Unfractionated bone marrow (4×10^8)	53	72
Unfractionated bone marrow (25×10^6)	4	2
Eluate (24×10^6)	39	8
Eluate (24×10^6) + unfractionated bone marrow (25×10^6)	47	3
Effluent (10^8)	6	44
Effluent (24×10^6)	3	4
Effluent (10^8) + unfractionated bone marrow (25×10^6)	7	61

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injections of 10^9 *S*-RBC and 10^9 *H*-RBC.

resulted in the retention of sheep red cell–reactive cells by the glass bead column (Table XII).

In order to ascertain whether the capacity of the bone marrow antigen–reactive cells to react with antigen on the glass beads is dependent on the presence of other "nonspecific" cell types (i.e. macrophages), which are them-

selves removed by the glass beads, normal bone marrow cells were passed through an unsensitized glass bead column, followed by passage of the recovered effluent cells through an SCS-sensitized column (Table XIII). The unsensitized column did not retain any cells capable of transferring specific plaque-forming capacity; on the other hand, a second passage of the cells through the SCS-sensitized column resulted in the specific retention of cells capable of mediating an immune response to sheep red cells (Table XIII).

TABLE XII

Plaque-Forming Capacity, with Respect to Sheep and Horse Red Cells, of Spleen Cells of Irradiated Rabbits Given Bone Marrow Cells Fractionated Initially by Passage through a Horse Red Cell–Sensitized Glass Bead Column Followed by Passage through a Sheep Red Cell–Sensitized Glass Bead Column

Normal bone marrow cells fractionated as follows	No. of cells in each fraction injected into irradiated recipient*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with	
		H-RBC	*S*-RBC
Unfractionated cells	Unfractionated cells (200×10^6)	80	72
↓ HCS column			
↓ Eluate cells	Eluate of HCS column (25×10^6)	69	8
Effluent cells	Effluent of HCS column (200×10^6)	9	52
↓ SCS column			
↓ Eluate cells	Eluate of SCS column (20×10^6)	0	39
Effluent cells	Effluent of SCS column (120×10^6)	2	5

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injections of 10^9 *S*-RBC and 10^9 *H*-RBC.

D. Morphology of Cells Recovered following Passage of Normal Bone Marrow Cells through an Antigen-Sensitized Glass Bead Column.—Normal bone marrow cell suspensions were passed through antigen-sensitized and unsensitized glass bead columns, and the fractions collected—the effluent and eluate fractions—and the original unfractionated bone marrow were compared on the basis of their morphological characteristics. The effluent cells appeared to be morphologically indistinguishable from the unfractionated bone marrow, both qualitatively and quantitatively (Figs. 3 and 4). The effluent consisted of all the erythrocyte and leukocyte precursors, the blast cells, macrophage-like cells, and mature erythrocytes and leukocytes. The eluate fraction of cells was far more uniform in composition (Fig. 5) and consisted of only small mononuclear cells.

DISCUSSION

In the present investigation, it has been demonstrated that passage of normal rabbit bone marrow cells through an antigen-sensitized glass bead column results in the retention of specific antigen–reactive cells capable of mediating an immune response in an irradiated recipient only toward the antigen used to sensitize the column. The cells which were not retained by the column (effluent) did not possess the capacity to transfer plaque-forming ability with

TABLE XIII

Plaque-Forming Capacity, with Respect to Sheep Red Cells, of Spleen Cells of Irradiated Rabbits Given Normal Allogeneic Bone Marrow Cells Fractionated by Passage of Cells through an Unsensitized Glass Bead Column Followed by Passage through a Sheep Red Cell–Sensitized Glass Bead Column

Normal bone marrow cells fractionated as follows	No. of cells in each fraction injected into irradiated recipient*	No. of plaques per 10^6 recipient splenic lymphoid cells incubated with *S*-RBC
Unfractionated cells	Unfractionated cells (46×10^7)	52
↓ Unsensitized column		
↓ Eluate cells	Eluate cells (18×10^6)	2
Effluent cells	Effluent cells (200×10^6)	39
↓ SCS column		
↓ Eluate cells	Eluate cells (18×10^6)	28
Effluent cells	Effluent cells (60×10^6)	3

* Rabbits were subjected to 800 R total body irradiation followed by the intravenous injections of 10^9 *S*-RBC.

respect to the specific red cell antigen used to sensitize the column, although they could facilitate a response to a non–cross-reacting red cell following their transfer to irradiated recipients. Furthermore, effluent cells obtained from an HSA-sensitized glass bead column could not transfer humoral antibody-forming capacity with respect to HSA when transferred to the irradiated host. On the other hand, effluent cells from a nonsensitized column were capable of conferring antibody-forming capacity toward HSA or sheep red cells in irradiated hosts, as were bone marrow cells retained by and eluted from the specific antigen–sensitized columns. The presence of residual antigen (i.e. HSA) in the medium bathing the antigen (HSA)–sensitized glass beads prevented the retention by the

column of cells capable of mediating an immune response to the antigen (HSA) in the irradiated host (Table VI). This probably was due to the competition by free antigen and glass bead-adsorbed antigen for the antigen-reactive cells, the interaction probably occurring between the antigen and an antibody-like site on the surface of the antigen-reactive cell (see below). These experiments demonstrated that cells are specifically retained by the antigen-sensitized glass bead columns, since unsensitized glass bead columns did not retain any of the specific antigen–reactive cells. Furthermore, the transfer of immunological activity by the eluted cells was shown to be specific, in that it was directed solely to the antigen originally used to sensitize the column. The converse is true for the effluent cells, since they were unable to transfer immunocompetence with respect to the antigen used to sensitize the glass beads. The magnitude of the immune response in the irradiated recipient given the eluate cells, which constitute 3–10% of the cells of the bone marrow, was on a level similar to that observed in recipients given the whole unfractionated bone marrow. This, in itself, is an unexpected finding when it is realized that as few as 20×10^6 eluate cells could confer an immune response in a recipient equivalent to that mediated by $200–500 \times 10^6$ unfractionated bone marrow cells. This demonstration of specific immunocompetence of the eluate cells is even more remarkable when it is realized that many lymphoid cells other than the specifically bound, antigen-reactive cells are nonspecifically retained by the glass beads and are subsequently eluted along with the antigen-reactive cells. Wigzell and Andersson (9) arrived at a similar conclusion. Therefore, although the technique may not permit the isolation only of specific antigen-reactive cells, it does make possible the complete segregation of one species of antigen-reactive cells from all the others. Stimulation of the cells by the specific antigen in vitro in the presence of tritiated thymidine, followed by radioautographic analysis of the cell preparation, may provide a more precise indicator of the number of specific antigen–reactive cells in the eluate fraction.

No significant differences were observed in the immune responses of irradiated recipients given eluate cells alone or eluate cells plus a subthreshold dose of the unfractionated bone marrow. The latter dose of bone marrow cells was incapable, by itself, of mediating an immune response in an irradiated recipient. The reasons for the administration of this dose of bone marrow cells were twofold: (*a*) to decrease the severity of irradiation-induced morbidity and/or mortality in sublethally irradiated rabbits receiving only a small number of bone marrow (i.e. eluate) cells (we have observed that approximately 10–20% of the irradiated rabbits die if they are not protected with bone marrow) and (*b*) to provide the recipient with macrophages and/or other cell types which, although not the predominant cell(s) required for the induction of the primary immune response, may nevertheless be necessary in small numbers in order to potentiate the immune response, or to function in a synergistic fashion. Macrophages have

been shown to play an active role in promulgating the sequential steps leading to humoral antibody formation, especially in the primary response (11–15). It has also been demonstrated that macrophages are retained by the glass bead columns (16–18) and apparently are not eluted by the procedure utilized in this investigation. Furthermore, the glass bead–purified lymphocyte suspensions have been found to display a markedly reduced response to antigens, which could be restored to normal by the addition of macrophages to the system (17, 18). It was therefore felt that the administration of a subthreshold dose of unfractionated bone marrow along with the test cell preparation (eluate or effluent) might enhance the primary immune response in the irradiated recipient.

The results, however, do not lend support to these theoretical considerations. The immune response of irradiated recipients given eluate cells and a subthreshold dose of unfractionated bone marrow cells was only slightly enhanced compared with that obtained in a recipient given eluate cells only. Furthermore, initial passage of the unfractionated cell suspension through a column sensitized with a non–cross-reacting antigen (Table XII) or an unsensitized glass bead column (Table XIII) prior to passage of the cells through a specifically sensitized column did not diminish the effectiveness of the eluate cells to transfer specific immunocompetence to irradiated recipient rabbits. Since the initial passage of the cells should have depleted the cell suspension of macrophages or seriously reduced their number (17–19), it is obvious that the functionally active macrophage does not participate in the interaction between the antigen-reactive cell and the antigen adsorbed onto the glass beads. Although one might be tempted to rule out any role for the macrophage in the induction of the primary response in the irradiated recipient rabbit, it would be necessary to establish unequivocally that the exposure to 800 R irradiation killed all the macrophages and their precursor cells in vivo and that no macrophages were transferred with the eluate cells. Although macrophages could not be distinguished morphologically in the eluate population of cells, it is possible, because of the manipulation of the cells, that they had assumed a different appearance. Furthermore, it has been demonstrated that lymphocytes can transform to macrophages (19), thus rendering academic the entire question of the role of the macrophage.

Glass bead columns have been utilized by a number of investigators in attempts to separate morphologically identical but functionally different lymphocyte populations (9, 16–18, 20–22). Plotz and Talal (16) passed *immune* mouse and rat spleen cells through glass bead columns and observed that only small, immunoincompetent mononuclear cells passed through, whereas the column retained the antibody-synthesizing cells, granulocytes, and large mononuclear cells, which could be eluted from the bead with ethylenediaminetetraacetate. Oppenheim et al. (17) and Hersh and Harris (18) have observed that after purification on glass beads human peripheral lymphocytes were capable

of reacting to stimulation by PHA (phytohemagglutinin) but could no longer be stimulated by a number of antigens to which the original donors had been actively immunized and to which the unfractionated cells could react. The immunological responsiveness of the lymphocytes could be reestablished by the addition of macrophages. Nossal et al. (22) also observed that column-purified small lymphocytes, prepared from *normal* mouse thoracic duct lymph, could not transfer immunocompetence to a number of antigens in irradiated recipients, whereas the unfractionated cell suspensions could transfer antibody-forming capacity. However, column-purified small lymphocytes obtained by fractionating *immune* mouse thoracic duct lymph could successfully transfer antibody-forming capacity to irradiated hosts. These findings suggest that the macrophage, which is normally retained by the glass bead column, is required along with the small lymphocyte (probably the antigen-reactive cell) in the initiation of the primary immune response (23). This cell, however, does not appear to be necessary for the induction of the secondary immune response, since the antigen apparently reacts directly with the antigen-recognizing–antibody-forming (memory) cell (24, 25).

Wigzell and Andersson (9) fractionated *immune* mouse lymph node cells, using a specific antigen–sensitized glass bead column. They observed that antibody-forming cells, directed toward the immunizing antigen, were retained by the glass beads and could subsequently be eluted from the beads by shaking. The binding of the cells to the antigen-sensitized glass beads was found to be selective for the particular cell population, since cells from an animal immunized to two different antigens were deprived of reactivity to only one of the antigens, that adsorbed onto the glass beads, following passage through the column. Thus, the specific interaction of the antigen-bead complex with the antibody-forming cell cannot be ascribed to passively adsorbed antibody by the cells. However, the great specificity of the interaction makes it mandatory to assume an immunological nature for the interaction, and it must be due to reaction of the glass-adsorbed antigen with specific antibody or antibody-like sites produced by, and retained on the surface of, the specifically retained immune cells themselves. This explanation might not be the only one, however, for it does not aid at all in elucidating the mechanism of interaction observed in the current investigation. Here *normal, not immune,* cells were utilized, and it was a select population of normal bone marrow cells which was retained by the antigen-sensitized glass bead column. Since no immune response had previously been induced in the donor of these cells, the interaction of the normal bone marrow cells with the antigen adsorbed onto the glass bead column cannot be attributed to conventional antibody. However, the specificity of the interaction between the antigen and a particular immunologically specific lymphoid cell necessitates the assumption of the presence of some sort of immunoglobulin or antibody-like structure on the surface of the cell capable of "recognizing" the antigen and of

reacting with it. This concept was originally presented by Ehrlich almost a century ago (26) as the "side chain theory," and has been discussed at length by Mitchison (27) and Gell (28). Recent investigations (29–31) tend to confirm the validity of this concept. It has been observed that goat anti-rabbit immunoglobulin antiserum (GARIG) can stimulate normal rabbit lymphocytes to undergo blastogenesis and mitosis in the same manner as antigen can stimulate immune lymphocytes.[1] Furthermore, rabbit peripheral lymphocytes incubated with GARIG are capable of conferring antibody-forming capacity directed to goat gamma globulin when transferred to recipient rabbits previously made tolerant to goat gamma globulin, whereas rabbit lymphocytes incubated with normal goat gamma globulin do not possess this activity.[1] These data suggest that GARIG exhibits a great affinity toward peripheral lymphocytes and imply that GARIG reacts with an immunoglobulin or immunoglobulin-like site on the surface of the cell. Paul et al. (32) have also demonstrated a relationship between the affinity of an antigen for lymphoid cells and the capacity of the antigen to induce mitosis of the cells in vitro. Fidalgo and Najjar (30) arrived at a similar conclusion in investigations with dog lymphoid cells. Merler and Janeway (29) have eluted a material from immune human lymphoid cells which was capable of interacting with the antigen, thus suggesting that it is an immunoglobulin. It would therefore appear that normal, as well as immune, lymphoid cells possess immunoglobulins or immunoglobulin-like structures on their surfaces, which exhibit specific affinities toward particular antigens.

The above interpretation goes a long way toward an understanding of the mechanism of interaction between the antigen-reactive cell and the antigen. Certainly, the reaction of the normal bone marrow cells with antigen cannot be attributed to interaction of the antigen with antibody-forming cells (which would be expected to possess specific immunoglobulin molecules on their surfaces), since previous investigations have disclosed that the immunocompetent cells residing in normal bone marrow are antigen-reactive cells and not antibody-forming cells (actual or potential) (8). Thus, it would appear that the antigen-reactive cell exercises its function on the basis of specific antibody-like sites on its surface capable of reacting with the antigen.

The data presented here, demonstrating a specific interaction between antigen and antigen-reactive cells in normal rabbit bone marrow, and the isolation of the latter cells, confirm previous findings concerned with the immunological role of the bone marrow (1–5, 8). It has previously been noted that normal rabbit bone marrow cells react with blastogenesis and mitosis as a result of in vitro stimulation with a great variety of antigens (1, 2), and that bone marrow cells obtained from a rabbit injected with the antigen only 8–24 hr prior to

[1] Daguillard, F., and M. Richter. Unpublished results.

sacrifice (primed bone marrow) lose the capacity to respond to the specific immunizing antigen in vitro, but not to other antigens (2). One explanation offered was that the immunocompetent antigen-reactive cells in the normal bone marrow emigrate from the bone marrow following contact with the antigen. However, the target organ (or organs) for these cells and their further functions in the immune response, if any, have not been elucidated (Fig. 2). Another, equally plausible theory is that the bone marrow cells become immunologically tolerant to the antigen following intravenous administration of the antigen and therefore cannot interact with the antigen in vitro. A third inter-

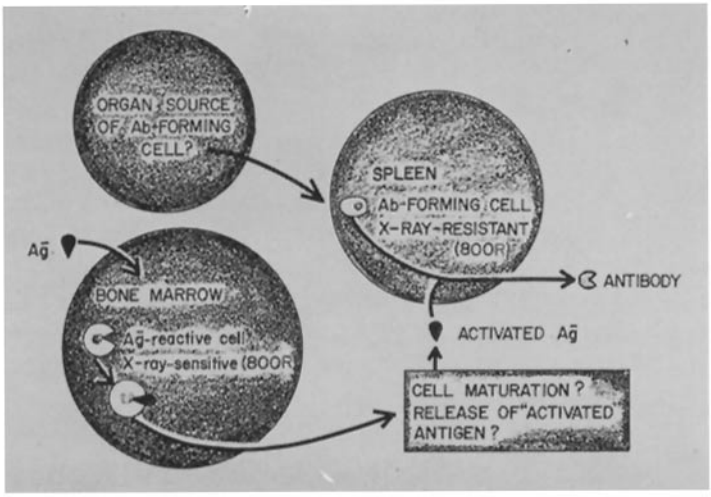

FIG. 2. Cellular interactions during the immune response in the rabbit; a diagrammatic representation.

pretation of our previous data is that the reaction of normal bone marrow cells with antigen in vitro has nothing to do with immunocompetence but is simply an incidental interaction facilitated by the conditions of the experiment. The findings reported in recent studies (8), demonstrating the antigen-reactive nature of the immunocompetent cells in normal bone marrow, and the present demonstration that the antigen-reactive cells in normal bone marrow interact with the antigen adsorbed onto glass beads and are capable of expressing immunocompetence following their elution from the glass beads, support only the first concept presented above. These data, therefore, strongly indicate that antigen-reactive cells in fact migrate out of the bone marrow in the rabbit following contact with the antigen. Since specific antigen-reactive cells do not repopulate the bone marrow even months following the immune response (2), one may assume that the bone marrow of an immune rabbit, but not any single

cell in the marrow, is in fact immunologically tolerant with respect to the specific immunizing antigen. Since the cells which vacate the bone marrow following antigenic stimulation appear to be directed to only a single particular antigen, it would appear that they all arise from a single precursor cell or clone of cells, thus leaving the bone marrow deficient in cells capable of responding to this antigen. This concept, therefore, strongly supports the clonal selection theory of Burnet, proposed more than a decade ago (33), and implicates the bone marrow as the organ in the rabbit which serves as the main source of the precursors or mature representatives of antigen-reactive cells. Investigations in the mouse, however, have not been as precise in elucidating the organ source of the antigen-reactive cell. In this regard, it has been demonstrated that irradiated mice are capable of an immune response only if given allogeneic bone marrow and thymus cells (34–39). Miller and Mitchell (38) have unequivocally demonstrated that the thymus cell, rather than the bone marrow cell, must first interact with the antigen in order for the immune response to ensue in the recipient irradiated mouse. These data suggest that the thymus in the mouse possesses the antigen-reactive cells (39). Taylor (34) has presented evidence that further corroborates this view. He observed that irradiated mice responded with antibody formation if given allogeneic bone marrow and thymus cells along with the antigen, bovine serum albumin (BSA). However, if the recipient irradiated mouse was given normal bone marrow cells and thymus cells from a donor injected with BSA 24 hr prior to sacrifice (primed thymus cells), no immune response was observed. On the other hand, irradiated recipients of primed bone marrow cells and normal thymus cells gave good immune responses. These data indicate that, in the mouse, the thymus possesses the antigen-reactive cells which, as in the rabbit, vacate the thymus after contact with the antigen in vivo, and that the bone marrow in the mouse constitutes the store of antibody-forming cells (39). Furthermore, since irradiated mice do not exhibit an immune response if given only thymus antigen-reactive cells and antigen (34–39), it would appear that the antibody-forming cell in the mouse is irradiation-sensitive, unlike that in the rabbit, which is irradiation-resistant (8). An alternative hypothesis, which must be ruled out experimentally, is that the antibody-forming cell in the mouse is of recipient origin and is irradiation-resistant to 800 R, as in the rabbit (8), but that another cell type, other than the thymic antigen-reactive cell, normally resident in the bone marrow is required in order to mediate the immune response in the mouse. The sequence of cellular events mediating the primary humoral immune responses in the rabbit and the mouse, as postulated by the present investigators, is presented in Fig. 2.

In summary, by the use of either of two techniques, one can obtain a population of normal marrow cells deficient in reactivity to a particular antigen. The methods are (*a*) extirpation of the bone marrow 8–24 hr following intravenous

administration of the antigen (primed bone marrow) (2) and (*b*) actual removal of the specific antigen–reactive cells from a suspension of normal bone marrow cells by passage of the cells through an antigen-sensitized glass bead column. The potential use of such specifically induced immunodeficient (tolerant) populations of cells is obvious, and experiments are currently underway utilizing immunologically induced immunoincompetent bone marrow cells in an attempt to inhibit the rejection of transplanted tissue in an allogeneic rabbit.

SUMMARY

The antigen-reactive cells in normal rabbit bone marrow could be isolated from a suspension of marrow cells by passage of the cells through an antigen-sensitized glass bead column. The cells which passed through the column were deficient in antigen-reactive cells directed to the antigen used to sensitize the glass beads, whereas the cells eluted from the column could transfer antibody-forming capacity to irradiated recipients only with respect to the specific sensitizing antigen. Separation of the bone marrow antigen–reactive cells could not be achieved by passage of the cells through nonsensitized glass bead columns or in the presence of excess free antigen in the column. Cells which were retained by, and later eluted from, the antigen-sensitized glass bead columns were mostly small mononuclear cells, whereas cells which passed through the columns were morphologically similar to the original unfractionated bone marrow cell suspension. The data indicate the presence of an antibody or antibody-like structure, with defined immunological specificity, on the surface of the normal bone marrow antigen–reactive cell.

BIBLIOGRAPHY

1. Singhal, S. K., and M. Richter. 1968. Cells involved in the immune response. I. The response of normal rabbit bone marrow cells to antigen in vitro. *Int. Arch. Allergy Appl. Immunol.* **33**:493.
2. Singhal, S. K., and M. Richter. 1968. Cells involved in the immune response. IV. The response of normal and immune rabbit bone marrow and lymphoid tissue lymphocytes to antigens in vitro. *J. Exp. Med.* **128**:1099.
3. Singhal, S. K., M. Richter, and D. G. Osmond. 1968. Cells involved in the immune response. III. Responsiveness to antigens of a lymphocyte-rich fraction of normal rabbit bone marrow. *Int. Arch. Allergy Appl. Immunol.* **34**:224.
4. Abdou, N. I., and M. Richter. 1969. Cells involved in the immune response. V. The migration of antigen-reactive immunocompetent cells out of the bone marrow following antigen administration. *Int. Arch. Allergy Appl. Immunol.* **35**:330.
5. Abdou, N. I., and M. Richter. 1969. 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. *J. Exp. Med.* **129**:757.
6. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for

recognizing antibody-producing cells. *In* Cell Bound Antibodies. Wistar Institute Press, Philadelphia. 109.

7. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Washington).* **140**:405.
8. Richter, M., and N. I. Abdou. 1969. Cells involved in the immune response. VII. The demonstration, using allotypic markers, of antibody formation by irradiation-resistant cells of irradiated rabbits injected with normal, allogeneic bone marrow cells and sheep erythrocytes. *J. Exp. Med.* **129**:1261.
9. Wigzell, H., and B. Andersson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* **129**:23.
10. Boyden, S. V. 1951. The absorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera. *J. Exp. Med.* **93**:107.
11. Gallily, R., and M. Feldman. 1967. The role of macrophages in the induction of antibody in X-irradiated animals. *Immunology.* **12**:197.
12. Argyris, B. F. 1967. Role of macrophages in antibody production. Immune response to sheep red blood cells. *J. Immunol.* **99**:744.
13. Argyris, B. F. 1968. Role of macrophages in immunological maturation. *J. Exp. Med.* **128**:459.
14. Ananue, E. R., and B. A. Askonas. 1968. The immune response of mice to antigen in macrophages. *Immunology.* **15**:287.
15. Adler, F. L., M. Fishman, and S. Dray. 1966. Antibody initiated in vitro. III. Antibody formation and allotypic specificity directed by ribonucleic acid from peritoneal cells. *J. Immunol.* **97**:554.
16. Plotz, P. H., and N. Talal. 1967. Fractionation of splenic antibody-forming cells on glass bead columns. *J. Immunol.* **99**:1236.
17. Oppenheim, J. J., B. G. leventhal, and E. A. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. *J. Immunol.* **101**:262.
18. Hersh, E. M., and J. E. Harris. 1968. Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leukocytes. *J. Immunol.* **100**:1184.
19. Rebuck, J. W., F. W. Whitehouse, and S. M. Noonan. 1967. Energization of lymphocytes transforming to macrophages in human inflammation. *Biochem. Pharmacol.* **16**(Suppl.):159.
20. Rabinowitz, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns including tissue culture observations. *Blood J. Hematol.* **23**:811.
21. Shortman, K. 1966. The separation of different cell classes from lymphoid organs. I. The use of glass bead columns to separate small lymphocytes, remove damaged cells and fractionate cell suspensions. *Aust. J. Exp. Biol. Med. Sci.* **44**:271.
22. 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.
23. Nachtigal, D., E. Greenberg, and M. Feldman. 1968. The kinetics of immune

tolerance to human serum albumin induced in sublethally X-irradiated rabbits. *Immunology*. **15**:343.

24. Richter, M. 1969. Cells involved in the immune response. XIII. Speculations concerning the nature of the cellular interactions relating antibody formation and immunologic tolerance. A unified hypothesis. In preparation.
25. Sercarz, E. E., and V. S. Byers. 1967. The X-Y-Z scheme of immunocyte maturation. III. Early IgM memory and the nature of the memory cell. *J. Immunol.* **98**:836.
26. Marquart, M. 1949. Paul Ehrlich. William Heinemann Medical Books Ltd., London. 65.
27. Mitchison, N. A. 1967. Antigen recognition responsible for the induction in vitro of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32**:431.
28. Gell, P. G. H. 1967. Restrictions on antibody production by single cells. *Cold Spring Harbor Symp. Quant. Biol.* **32**:441.
29. Merler, E., and C. A. Janeway. 1968. Immunological identification of cytophilic antibody in human lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* **59**:393.
30. Fidalgo, B. V., and V. A. Najjar. 1967. The physiological role of the lymphoid system. III. Leucophilic gamma-globulin and the phagocytic activity of the polymorphonuclear leukocyte. *Proc. Nat. Acad. Sci. U.S.A.* **57**:957.
31. Naspitz, C. K., and M. Richter. 1968. The action of phytohemagglutinin in vivo and in vitro. A review. *Progr. Allergy*. **12**:1.
32. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1968. Specificity of cellular immune responses. Antigen concentration dependence of stimulation of DNA synthesis in vitro by specifically sensitized cells, as an expression of the binding characteristics of cellular antibody. *J. Exp. Med.* **127**:25.
33. Burnet, F. M. 1958. The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville, Tenn.
34. Taylor, R. B. 1968. Immune paralysis of thymus cells by bovine serum albumin. *Nature (London)*. **220**:611.
35. Davies, A. J. S., E. Leuchars, V. Wallis, R. Merchant, and E. V. Elliot. 1967. The failure of thymus-derived cells to antigenic stimulus. *Transplantation*. **5**:222.
36. Davies, A. J. S., E. Leuchars, V. Wallis, and P. C. Koller. 1966. The mitotic response of thymus-derived cells to antigenic stimulus. *Transplanation*. **4**:438.
37. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Immune competence of transferred thymus-marrow cell combinations. *J. Immunol.* **97**:828.
38. Miller, J. F. A. P., and G. F. Mitchell. 1967. The thymus and the precursors of antigen reactive cells. *Nature (London)*. **216**:659.
39. 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.

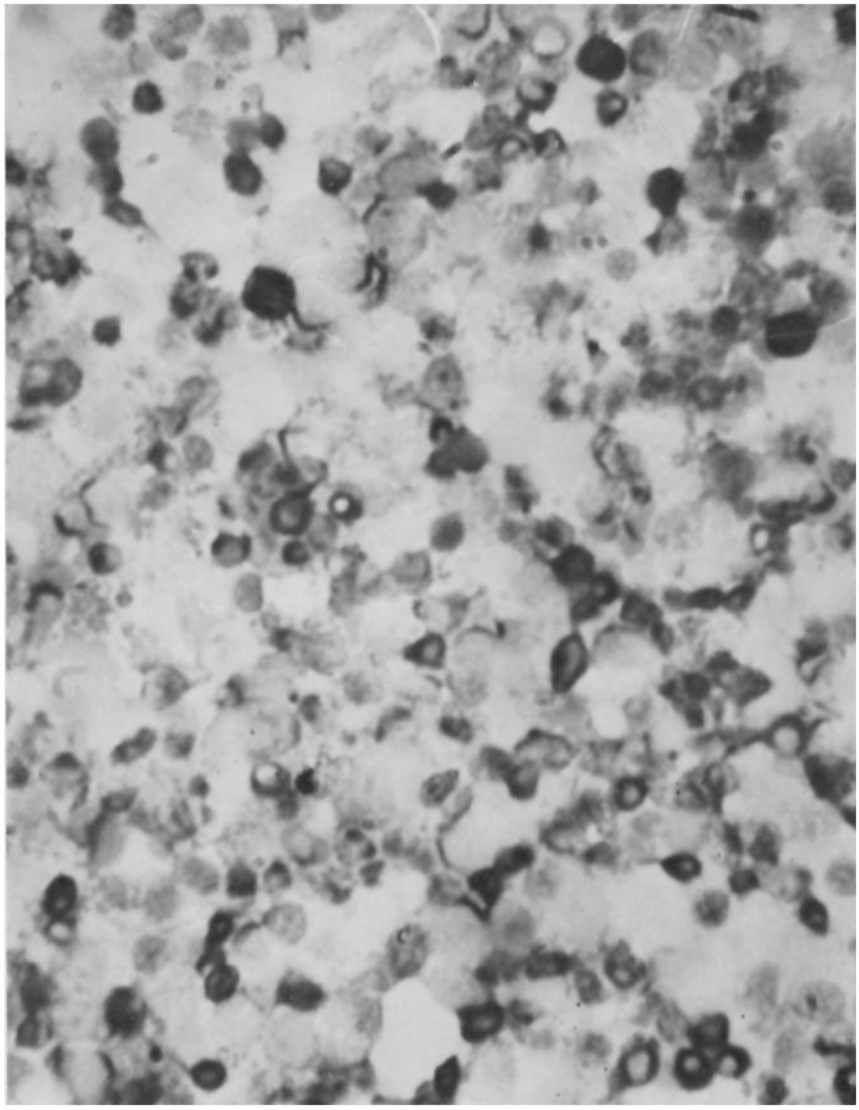

FIG. 3. Unfractionated bone marrow. Giemsa stain. × 640.

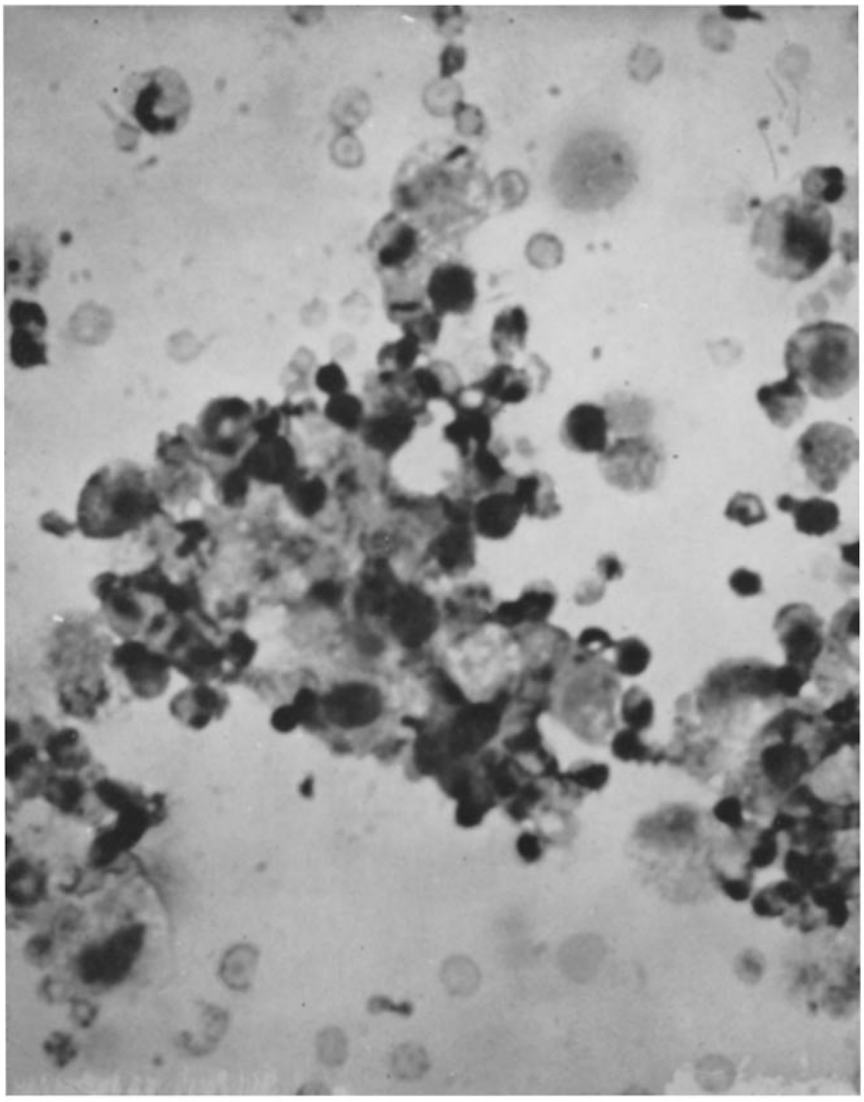

FIG. 4. Effluent fraction of cells. Giemsa stain. × 640.

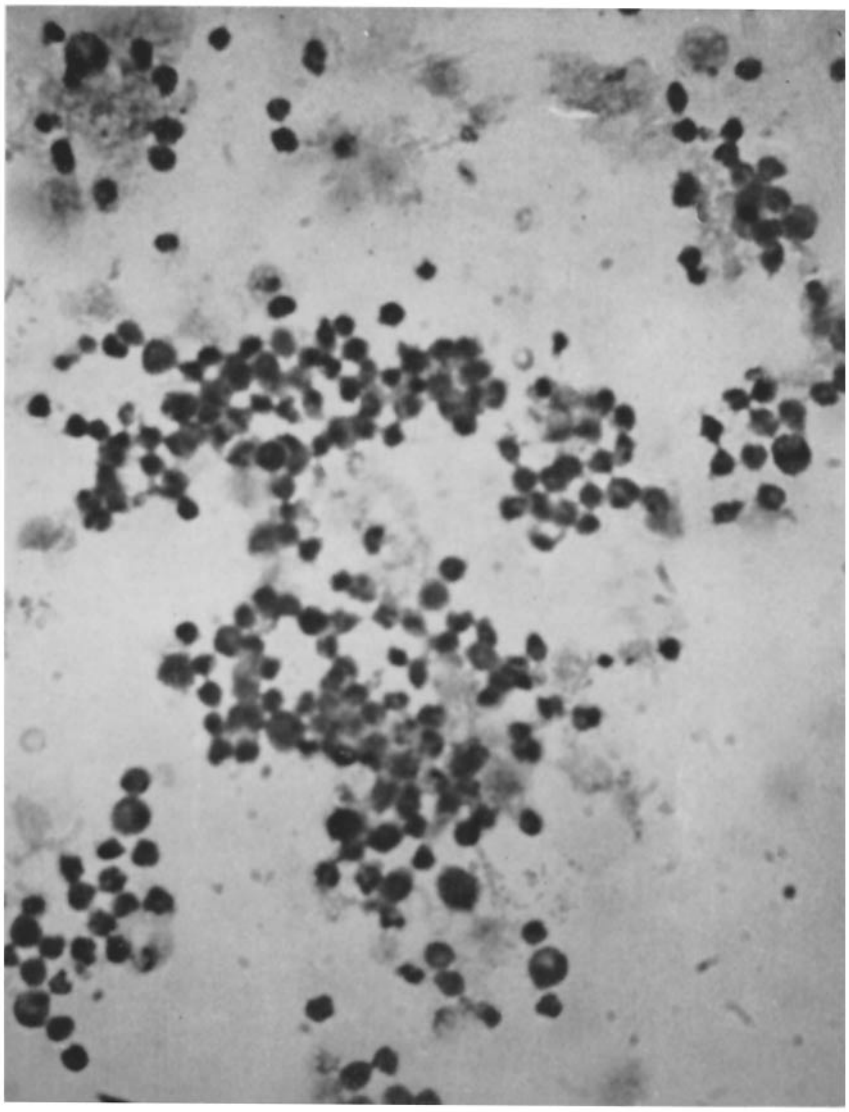

FIG. 5. Eluate fraction of cells. Giemsa stain. × 400.