CELLS AS ANTIGEN CARRIERS AND AS IMMUNOGLOBULIN PRODUCERS

Synthesis of Antibody and Allogeneic Immunoglobulin after Transfer of Antigen-Treated Cells to Newborn Rabbits

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Tolerance induction and antibody formation appear to be alternative consequences of exposure to antigen. The decision between the alternatives depends, on the one hand, on the dose (1, 2), structure (3), and molecular weight (4, 5) of the antigen and, on the other, on the age, species, and immunological status of the recipient. While some of the factors which cause tolerance in adult animals have been explored in detail, little is known about the conditions which promote antibody formation after neonatal exposure to an antigen. To explore this further, we have injected newborn rabbits with small quantities of human albumin (HA) either in soluble form or in association with rabbit cells. The protein was either linked to rabbit erythrocytes by covalent bonds or taken up by nucleated cells from the thymus, the lymph nodes, the peritoneal cavity, or the lungs. The cellular origin of antibody was determined by allotypic markers on the antibody molecules. The fate of the transferred cells in the recipient animal was followed by monitoring the allotypic markers of the circulating immunoglobulin molecules.

Materials and Methods

Glycogen.—Glycogen of cp grade was obtained from Pfanstiehl Chemical Co., Waukegan, Ill., and Fish Reagent Chemical Glycogen was obtained from Fish Scientific Co., New York. A 1% (w/v) stock solution of glycogen in glass-distilled water was prepared and was sterilized by steaming at 100°C for 30 min daily for 3 consecutive days. Immediately before use a 0.1% solution was prepared from this sterile stock solution by adding 100 ml of the 1% solution to 900 ml of Baxter sterile nonpyrogenic normal saline (Baxter Laboratories of Canada, Ltd., Alliston, Ontario).

Trypan Blue.—Trypan blue of BDH Standard Stain grade was obtained from British Drug Houses, Ltd., London, England. A 1% (w/v) solution was prepared in 0.15 M NaCl and was filtered.

Benzidine.—Benzidine of reagent grade was obtained from Anachemia Chemicals, Ltd., Montreal, Canada.

2-Mercaptoethanol (ME).—ME was obtained from Eastman Organic Chemicals, Rochester, N.Y.

Heparin.—Heparin was obtained from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada. It contained 1000 IU (USP) or approximately 10 mg/ml.

Radioactive Iodine (^{131}I).—^{131}I was obtained from Charles E. Frosst and Co., Montreal, Canada, as sodium iodide in aqueous solution free of cysteine.

Alsever's Solution.—This solution was prepared by dissolving 5.125 g of glucose, 1.05 g of NaCl, and 2.0 g of sodium citrate in glass-distilled water and by adjusting the volume to 250 ml; the pH was adjusted to 6.1 with crystals of citric acid.

Medium CMRL-1066.—The composition of this medium is identical with medium No. 858 (6, 7), except that Armour's (porcine) liver coenzyme concentrate is used at a level of 10 mg/100 ml in medium CMRL-1066 in place of six highly purified coenzymes included in medium No. 858, and the fat-soluble vitamins (A, D, E, and K) and ferric nitrate included in medium No. 858 have been omitted from medium CMRL-1066. Also, Five B vitamins present in earlier media, but omitted from medium No. 858, have again been added. The medium contains 0.02 mg % *n*-butyl *para*-hydroxybenzoate, which offers protection from certain molds; other antibiotics have been omitted (G. M. Healy, and R. C. Parker, unpublished experiments).

Nutrient Broth.—Bacto Nutrient Broth (dehydrated) was obtained from Difco Laboratories Inc., Detroit, Mich. A 4% (w/v) solution of this powder was prepared in 0.15 M NaCl. The solution was autoclaved at 15 lb (121°C) for 15 min.

Eagle's Basal Medium.—The medium was prepared according to Eagle (8).

Fine Stainless Steel Gauze.—Stainless steel Greening mesh cloth, 100 × 100 mesh, 0.0044 in. wire, was obtained from Greening Wire Co., Ltd., Hamilton, Ontario, Canada.

Reagents for Agglutination of Tanned Sensitized Sheep Erythrocytes.—Details have been previously described (9).

Human Serum Albumin (HA).—HA ("reinst," Behringwerke) was used as an antigen throughout the experiments.

Iodinated Human Serum Albumin (HA.^{131}I).—HA was trace-labeled with ^{131}I using the method given in references 10 and 11. The iodinated human albumin contained 0.5–1.4 atoms of iodine per molecule of human albumin.

Micro-Kjeldahl Assay.—Method and reagents have been previously described (12).

Determination of the Viability of Nucleated Cells.—The viability of cells was judged by their ability to exclude dye from their interior (13). Trypan blue was used to determine in this way the viability of nucleated cells. Cells were first suspended in a 5% (v/v) solution of normal rabbit serum in 0.15 M NaCl. One volume of this suspension was then added to an equal volume of a filtered solution of 1% (w/v) trypan blue in 0.15 M NaCl. Immediately after this addition the cells were examined under a microscope. Stained and unstained cells were counted. Cells with stained nucleus were assumed to be dead.

Elimination of Labeled Antigen from the Circulation of Rabbit.—HA.^{131}I (1.9 mg N per kg body weight) was injected intravenously into rabbits which had been previously given drinking water containing KI. The injected animals were bled daily, and the radioactivity in 0.05 ml of serum was measured. The protein-bound iodine was determined as previously described and all measurements were corrected for radioactive decay (11). The logarithms of final corrected radioactive counts per 0.05 ml of serum were plotted as a function of time in hours after the injection of HA.^{131}I.

We referred to diphasic elimination when only the phase of distribution and the phase of metabolic breakdown was observed and referred to triphasic elimination when immune elimination was observed.

We determined t from a plot of time versus logarithm of antigen concentration; and defined it as the time in hours of the intersection between the line joining all points of the second

phase with a line joining all points of the third phase. The t, defined here, is several hours shorter than the value $t_{0.1}$, defined by Cinader, St. Rose, and Yoshimura (14).

Cell Preparations.—

Thymus cells were obtained from thymus glands of 6–7 wk old rabbits. The glands were dissected, placed in a small amount of Eagle's medium, cut with scissors, and squeezed through stainless steel gauze. Usually more than 90% of the cells in a preparation were lymphocytes.

Lymph node cells: Mesenteric lymph nodes were dissected out and the cells were separated by a technique similar to that used to obtain thymus cells. 90% of the cells in a preparation so obtained were lymphocytes.

Spleen cells were separated by a technique similar to that described for thymus cells.

Bone marrow cells were obtained from the femurs by cutting off the epiphyses and rinsing the inside of the diaphysis with Eagle's medium. The bone marrow cells were then squeezed through stainless steel gauze.

L. cells (15) were cultured for 72–84 hr in medium CMRL-1066 (see Culture Media) supplemented with 10% horse serum. The cells were then centrifuged at 4°C and 800–1000 rpm for 6–8 min. The supernatant was decanted from the resulting packed cells. The stained cells (using Leishman's stain) were all big and round with pale cytoplasm and a dark nucleus.

Alveolar mononuclear cells from rabbits were obtained by the method of Myrvik, Leake, and Fariss (16). The thoracic cavity was opened and the upper part of the trachea was dissected free and clamped shut with a hemostat to avoid entrance of blood into the lung when the trachea was cut above the point where it was clamped. Lungs were then carefully separated from the other organs with the trachea still clamped shut. The lungs were washed with warm (37°C) 0.15 M NaCl, sponged free of excess fluid with gauze pads, and suspended by attaching a hemostat to the wall of the trachea leaving the lumen open. Eagle's basal medium was injected into the trachea until the lungs appeared distended, which usually required approximately 30–40 ml of Eagle's basal medium. The trachea was clamped off, the lungs were massaged gently, and the injected fluid was drained into a large test tube. A second aliquot of Eagle's basal medium was injected and recovered in the same way. The two cell suspensions were pooled and centrifuged at 4°C and 800–1000 rpm for 6–8 min. The supernatant was decanted from the resulting packed cells of which more than 90% were monocytes.

Rabbit erythrocytes were obtained from two or three donors. Blood was taken from the ear vein of adult rabbits and shed into Alsever's solution. The blood was centrifuged at 4°C and 1500 rpm for 5 min. Supernatant fluid and white cells were then removed. The packed red cells were washed twice in 0.15 M NaCl.

Mononuclear peritoneal exudate cells: Adult rabbits were injected intraperitoneally with 100 ml of sterile nutrient broth (Bacto Difco) in 0.15 M NaCl. 46–50 hr later the peritoneal cavity was opened and rinsed with 200 ml of chilled Eagle's medium, containing 1000 IU of heparin. Cells were separated from the fluid by centrifugation. Usually, 60–80% of the cells in a preparation were monocytes.

Polymorphonuclear leukocytes were obtained from adult rabbits. The animals were injected intraperitoneally with 200 ml of 0.1% glycogen (17); 4 hr later, 100 ml of heparinized Eagle's basal medium was injected into the peritoneal cavity. The abdomen was gently massaged and a gauge #15 needle, perforated on the surface perpendicular to the long axis of the cylindrical hollow, was introduced into the peritoneal cavity. The fluid was finally allowed to flow out through the needle without pressure or suction. The fluid was then filtered through fine stainless steel gauze and was centrifuged at 4°C and 1000–1200 rpm for 6–8. min.

Uptake of HA by Nucleated Cells.—The general conditions for antigen uptake in vitro were selected after exploratory experiments. In these tests the survival of cells was evaluated by the relative cell number before and after 10 washings of the incubated cells. Judged by these criteria, the cell number obtained after 10 washings was not significantly different whether

the cells had previously been incubated at 0°C for 15 min or for 120 min, and was not significantly different if the cells were incubated at 37°C for 5 min, or for 30 min. The uptake after 2 hr was, however, 10 times greater at 37C° than at 0°C. If cells were incubated at 37°C for periods exceeding 30 min the number of cells found after 10 washings decreased markedly as a function of time so that after 2 hr incubation at 37°C about 40% of cells were destroyed. On the basis of the foregoing observations, incubation times of 30 min at a temperature of 37°C were chosen for further experiments.

When 1×10^8–4×10^8 rabbit mesenteric lymph node cells were incubated with various

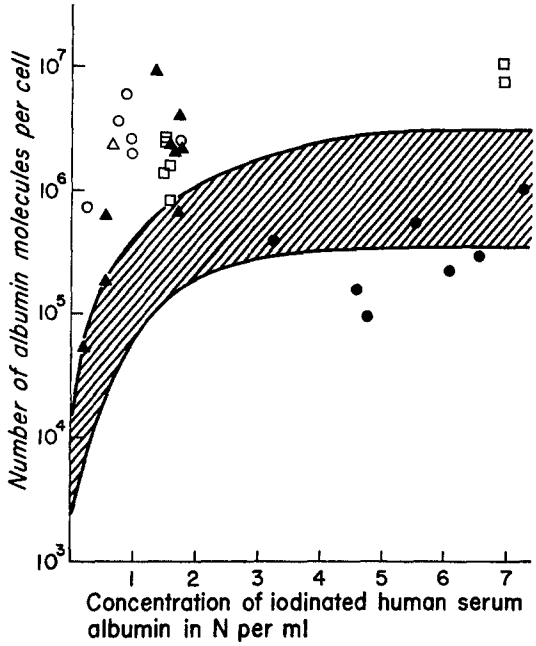

FIG. 1. Uptake of radioactively labeled human serum albumin (HA.^{131}I) by nucleated cells after incubation (37°C, 30 min) with different concentrations of HA.^{131}I. ○, Mononuclear peritoneal exudate cells. ●, Thymus cells. △, Pulmonary alveolar mononuclear cells. ▲, Polymorphonuclear leukocytes. □, Spleen cells. Uptake of HA.^{131}I by mesenteric lymph node cells is indicated by the shaded area.

amounts of HA.^{131}I in a final volume of 1 ml, the antigen uptake by the cells increased with increasing amounts of antigen until a point was reached (about 3 mg N of HA.^{131}I per ml), where further increase in antigen would *not* bring about a proportional increase in antigen uptake.

Fig. 1 shows the uptake of HA.^{131}I by rabbit mononuclear peritoneal exudate cells, polymorphonuclear leucocytes, thymus cells, spleen cells, and pulmonary alveolar mononuclear cells in comparison with the uptake by rabbit mesenteric lymph node cells (shaded area in Fig. 1) when 1×10^8–4×10^8 of these cells were incubated with various amounts of HA.^{131}I in a final volume of 1 ml. The antigen uptake by all these cells, except thymus cells, was higher than that by mesenteric lymph node cells. The antigen uptake by thymus cells was slightly lower than that by mesenteric lymph node cells. The antigen uptake by mononuclear peritoneal exudate cells seemed to be higher than that by any other cells.

On the basis of the foregoing observation, different incubation conditions were adopted for different cell types as shown in Table I. The general procedure was as follows:

Cells suspended in phosphate saline buffer (pH 7.5) were mixed with HA.^{131}I which was dissolved in the same buffer. The mixture was kept in a water bath at 37°C for 30 min with frequent gentle manual shaking. After this incubation the cells were chilled in an ice-water bath and were then centrifuged at 4°C and 800–1200 rpm for 4–6 min. The supernatant was carefully decanted and the packed cells were washed 10 times with the original volume of chilled Eagle's basal medium. Centrifugations were carried out at 4°C and 800–1200 rpm for 4–6 min. The cells were finally resuspended in Eagle's basal medium. Samples were taken for cell counts, determination of viability, differential cell counts, and determination of the cell content of HA.^{131}I.

Sensitization of Rabbit Erythrocytes with HA.—Bis-diazotized benzidine was prepared according to Stavitsky and Arquilla (18). 3 ml of phosphate saline buffer (pH 7.5) containing 4 mg of HA.^{131}I was mixed with 0.1 ml of a 50% (v/v) suspension of three-times-washed rabbit erythrocytes in chilled phosphate saline buffer (pH 7.4). To this mixture was added 0.6 ml of phosphate saline buffer containing 1.25 mg of freshly thawed bis-diazotized benzidine; after

TABLE I

*Conditions of Incubation of Nucleated Cells with Iodinated Human Albumin (HA.^{131}I)**

Cell type	HA.^{131}I	Number of cells ($\times 10^7$)
	(*mg N*)	
Monuclear peritoneal exudate cells	0.66–2	2.5–15
Polymorphonuclear leukocytes	0.5–1.8	12–35
Lymph node cells	1.7–6.6	20–54
Thymus cells	3.25–7.5	16–27
Alveolar mononuclear cells	0.3	5
L cells	0.16–0.18	4.4–6

* Amount of HA and number of cells in 1 ml incubation mixture.

the mixture had remained at room temperature for 15 min, the erythrocytes were washed three times and samples were taken for cell counts and determination of the cell-associated radioactivity.

Injection of Newborn Rabbits with Antigen-Associated Cells.—Nucleated cells were obtained from several donors and incubated with HA.^{131}I immediately before injection. Newborn animals were usually given 8×10^7 nucleated cells or of 8×10^8 rabbit erythrocytes in two intraperitoneal injections. The first injection was given within 24 hr and the second injection within 48 hr after birth.

Assay of Allotypically Defined Immunoglobulins—The quantity of allotypically defined immunoglobulins was estimated by two techniques: hemagglutination inhibition and single diffusion. The first of these two techniques employed allotypically defined rabbit immunoglobulins separated from serum by the method of Kekwick (19) and conjugated to sheep red cells by bis-diazotization according to Stavitsky and Arquilla (18). Test fluids (usually sera) which were assayed for allotype concentration were diluted in Microtiter trays, with 0.2% bovine albumin in 0.15 M phosphate buffer saline (pH 7.4). Equal volumes (0.025 ml) of anti-allotype immune serum were then added to the cups containing the dilutions. The immune serum contained antibodies directed against the allotypic specificity, to be assayed. Before addition to the test system, it was so diluted as to agglutinate sheep red cells sensitized with

the corresponding allotypically defined immunoglobulin with a titer 1:4 to 1:8. The mixtures of test fluid, diluent, and antiserum were incubated at 37°C for 1 hr and 0.025 ml of sensitized sheep red cells was added to each cup. The dilution of the test fluid, giving visible inhibition of the three-plus hemagglutination pattern, was considered as the titer of the given allotypic specificity.

The second technique for assaying allotypically defined immunoglobulins was that of single diffusion (20), modified as described previously (21, 22).

"Purification" of HA Antibodies by Immunoabsorbent.—The immunoabsorbent was prepared by conjugating HA to dispersed aminocellulose (23).[1] 0.5 ml of the tested serum was mixed with 0.4 mg of immunoabsorbent, shaken overnight at 4°C, then washed thoroughly with pH 7.4 buffered saline. Elution was performed with 0.5 ml of pH 3.0 glycine buffer; the samples were shaken for 15 min at room temperature. After centrifugation, the supernatants were collected and adjusted to pH 7.2–7.4 (bromothymol blue was used as an indicator) by adding 0.5 ml of pH 7.4 phosphate buffer and a small volume of 1 M NaOH. The quantity of antibody recovered from the immunoabsorbent was generally between 25% and 100% of the total antibody present in the serum before absorption. For sera from 2–3 wk old animals the recovery was usually lower (5–25%).

Determination of the Cellular Origin of Antibody.—Rabbits belonging to one allotypic group (A4 or A5) were injected with nucleated cells which had been incubated with HA.^{131}I. Injections were given 24 hr and 24–48 hr after birth. The cells were obtained from donor rabbits which differed with respect to their allotypic group (A5 or A4, respectively). Young recipient animals were bled at weekly intervals for 20 wk starting from the age of 14 days. Each serum sample was then divided into two parts: the first was left untreated and we will refer to these samples as "original" samples. The second part was absorbed with HA-conjugated immunoabsorbent. As a result of the absorption two more samples were obtained: (*a*) "eluate" and (*b*) "supernatant," i.e., the serum sample recovered after the absorption with immunoabsorbent. All these samples were next assayed for the presence of antibodies to HA as well as for the presence of both types of allotypically defined immunoglobulins (A4 and A5). As a rule, the absorption and titration procedures of all serum samples from one rabbit were carried out in the same experiment.

RESULTS

Immune Status of Neonatally Injected Animals.—Newborn rabbits were injected intraperitoneally with 1 to 3 μg N of human albumin (HA), either in solution, attached to red cells (8×10^8), or taken up by live nucleated (8×10^7) cells. When the animals were 68–78 days old, their immune status was determined by the mode of elimination of iodinated human albumin (Table II). In 98 out of 100 animals the iodinated albumin (HA.^{131}I) was eliminated in three phases, indicating that an immune response had occurred; the two animals (2/15; Table II) in which elimination occurred in two phases had been injected with HA in solution (see below). We also carried out similar elimination tests on the 30th day with seven animals injected with HA at birth and in this group there were also two animals which eliminated HA diphasically. It has been shown on a previous occasion (14) that the time at which the immune phase of elimination occurs can serve as an additional indicator for the immune

[1] Sehon, A. Personal communication.

status of a group and that the value of about 250 hr is the shortest period at which this occurs in animals which have lost tolerance after an exposure to antigen at birth (14). In the present series, the time at which a third phase of elimination started was the same in animals not injected at birth and in animals injected with soluble HA at birth, but it was significantly shorter among all the animals which received HA attached to or ingested by cells. Thus, soluble HA induced tolerance or left the immune status unaffected, whereas the cell-associated HA modified the immune apparatus so as to lead to accelerated response to a second exposure to the protein (Table II).

Presence of Antibody in the Serum of Neonatally Injected Rabbits.—The effect

TABLE II

The Inception of the Immune Phase Elimination in Animals not Injected at Birth and in Animals Injected at Birth with HA in Solution or Attached to Cells*

Animals injected with	Amount of HA injected at birth	Number of rabbits	t after challenge at the age of 68–78 days	Comparison with animals not injected at birth
	(μgN)		(hr)	
Not injected	0	26	260 ± 16	
HA	2.8	13‡	225 ± 24	$0.2 < P < 0.30$
HA + RBC*	0.6–1.8	25	189 ± 9	$0.01 < P < 0.02$
HA + polymorphonuclear leukocytes	0.8–2.8	11	196 ± 17	$0.001 < P < 0.01$
HA + lymph node cells	0.6–2.3	12	169 ± 13	$P < 0.001$
HA + thymus cells	0.5–0.9	13	126 ± 15	$P < 0.001$

* HA, human albumin; RBC, rabbit red blood corpuscles.

‡ Of 15 animals, 2 eliminated HA.^{131}I diphasically. The t value, given here refers to the remaining 13 animals which eliminated HA.^{131}I triphasically. A further seven animals were injected with HA.^{131}I on the 30th day of life and two animals eliminated the antigen diphasically (see also p. 310). The average t value for the remaining five animals was 216 ± 1 hr.

of neonatal injections was further examined by determining the agglutination titer in the serum of neonatally injected animals at regular intervals between the 12th and 68th day. None of the 36 animals injected with HA in solution formed detectable antibody but a proportion of all the animals injected with the same protein associated with cells did form antibody. The proportion of antibody formers was lowest among the animals which received HA attached to erythrocytes or taken up by polymorphonuclear leukocytes and was highest among animals which were given HA taken up by thymus cells, mononuclear peritoneal exudate cells, or lymph node cells (Table III). The antibody response of the animals neonatally injected with antigen attached to erythrocytes or in association with polymorphonuclear leukocytes was characterized by long delay and short duration of antibody (Table III). The average titer of antibodies to HA

varied, according to treatment, in the same sequence as the incidence of antibody formers. The highest titer of responding animals was found among animals injected with HA associated with lymph node cells and thymus cells and was slightly lower among animals injected with the protein taken up by mononuclear peritoneal exudate cells. It was markedly lower in the animals injected with HA taken up by polymorphonuclear leukocytes (Fig. 2).

The time course of the appearance and of the quantity of antibody showed considerable variants among animals neonatally injected with antigen in association with the same cell type. Sometimes a peak titer was already established when the first bleeding was taken and thereafter the titer declined slowly and then increased again. With other animals, the titer reached a peak after 4 to 5

TABLE III

Fraction of Responders among Animals Injected at Birth with HA in Solution or Attached to Rabbit Cells

Animals injected with	Fraction of animals having circulating antibody on day:						
	12–16	18–24	25–33	33–40	42–49	49–68	12–68*
HA	0/36	0/36	0/36	0/33	0/33	0/33	0/36
HA + Erythrocytes	3/80	5/80	3/78	2/73	12/72	13/71	20/80
HA + Thymus cells	18/58	40/58	36/57	30/55	33/54	34/49	45/58
HA + Lymphnode cells	37/104	58/103	59/100	57/99	60/94	61/92	75/104
HA + Mononuclear peritoneal exudate cells	50/91	67/90	77/91	74/91	29/35	76/87	81/91
HA + Polymorphonuclear cells	7/30	11/29	16/30	13/30	15/28	15/26	21/30

* In this column, an animal is considered as a responder if it had produced antibody (reciprocal titer $\geqslant$ 10) during any one of the six observation periods.

wk, declined and reached a second peak much later; in yet other animals a single peak of antibody titer was observed (Fig. 3). These three types of response were observed in animals given antigen associated with lymph node cells, thymus cells, and mononuclear peritoneal exudate cells. The type of response shown in Fig. 3 *B* was seen most frequently when thymus cells were administered.

The cell populations so far described were not the only ones which helped the induction of antibody formation. Pulmonary alveolar mononuclear cells also had this capacity though we encountered difficulties in demonstrating this.

Four animals were injected (intraperitoneally) at birth with 8×10^7 albumin-treated pulmonary alveolar mononuclear cells given in two injections. The animals died soon after the last injection. Another group of eight animals received in one injection less than 24 hr after birth 0.9 μg N HA associated with 4×10^7 cells. Two of these animals survived; one animal formed demonstrable antibody

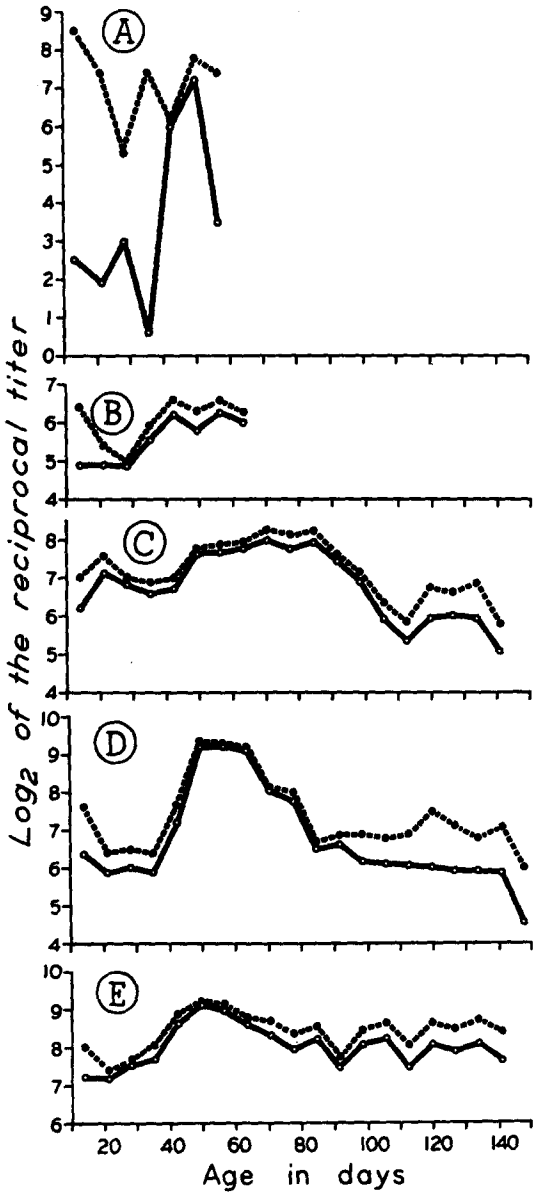

FIG. 2. Average reciprocal titers of antibodies to HA in various groups of experimental rabbits. *A*, Average reciprocal titer of rabbits injected with HA covalently linked to sheep red cells. *B*, Average reciprocal titer in serum of rabbits injected with HA, taken up by polymorphonuclear leukocytes. *C*, Average reciprocal titer in the serum of rabbits injected with HA, taken up by mononuclear peritoneal exudate cells. *D*, Average reciprocal titer in the serum of rabbits injected with HA, taken up by lymph node cells. *E*, Average reciprocal titer in the serum of rabbits injected with HA, taken up by thymus cells. The full line shows the average reciprocal titer of the serum of all the animals. The broken line shows the average reciprocal titer in the serum of all the responding animals.

first in the 3rd wk with a reciprocal titer of 100 which rose to 200 in the following week. The reciprocal titer remained at 200 until the 6th wk, then declined to 100 in the 7th wk, and was no longer detectable in the 8th wk.

It would seem as if some factor, associated with pulmonary alveolar mononuclear cells, possibly an infective agent, had a lethal effect on newborn animals. Nevertheless, it is apparent that pulmonary alveolar mononuclear cells, just as mononuclear peritoneal exudate cells, can promote antibody formation.

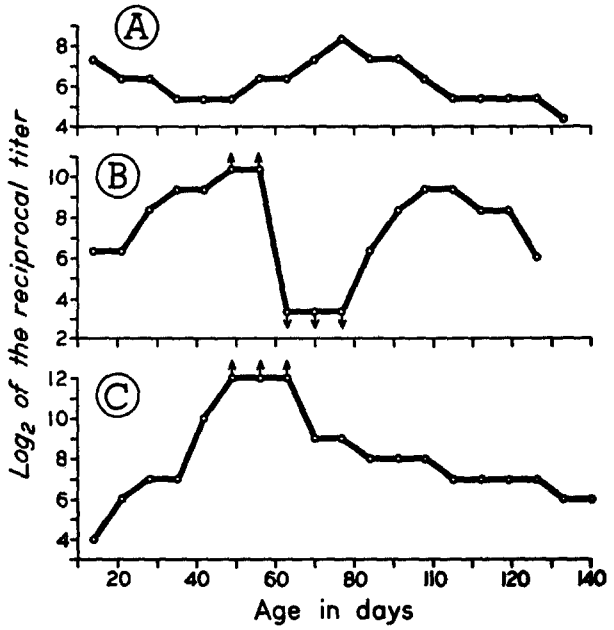

FIG. 3. Kinetics of antibody response to HA as a result of injection of HA, taken up by lymphoid cells. *A*, Two wave response with early first wave (Rabbit No. 32-3B injected with mononuclear peritoneal exudate cells). *B*, Two wave response with late first wave (Rabbit No. 54-0A injected with thymus cells). *C*, One wave response (Rabbit No. 21-41G injected with lymph node cells.)

We turned next to the question as to the properties of cells which were decisive in the induction of antibody response in very young animals.

Promoting Capacity of Nucleated Cells.—Was the attachment of antigen to cells the critical factor in the induction of antibody formation, or did the transplanted cells act in some way to render newborn animals capable of making antibody to HA? To answer this question, 28 newborn rabbits were given 8×10^7 untreated mononuclear peritoneal exudate cells in two intraperitoneal injections less than 24 and 48 hr after birth. Each injection was followed, 10 min later, by the intraperitoneal administration of HA (1.4 μg N) in solution. None

of the animals made demonstrable antibody during the observation period of 2 months. We examined the immune status of some of these animals by following the elimination of HA.^{131}I, injected intravenously 68 days after birth. Two of the eight animals showed diphasic elimination (compare with Table II); the remaining six animals showed triphasic elimination with an average value of $t = 278 \pm 20$ hr. This was not significantly different ($0.5 < P$) from the average value for t (260 ± 16 hr) of 26 control animals (Table I), not injected at birth. Thus the immune response of animals receiving at birth HA only and of animals receiving mononuclear peritoneal exudate cells in addition to HA was indistinguishable (see Table IV). It may, therefore, be concluded that adult mononuclear cells did not have any modifying effect on the immune apparatus of

TABLE IV

The Response to Neonatal Injection of HA Given Alone or Accompanied by a Separate Injection with Nucleated Cells

Reponse	Animals receiving at birth:		
	HA *only*	Mononuclear cells and HA (*separately*)	Comparison between I and II
	I	II	
Fraction of responders*	0/34	0/28	
Fraction of animals showing diphasic elimination when tested 10 wk after birth	2/15	2/8	$0.9 < P$
Average t of animals showing triphasic elimination	225 ± 24 hr	278 ± 20 hr	$0.1 < P < 0.5$

* Fraction of animals which produce agglutinating antibody.

newborn animals and that the crucial factor in the induction of antibody formation in very young animals was attachment of HA to mononuclear cells.

Having established that the association between mononuclear cells and HA was a necessary condition for inducing antibody formation in very young animals, we turned to another problem. We asked ourselves whether induction of antibody formation depended on the viability of albumin-treated nucleated cells? To answer this question, newborn animals were injected with heated HA-treated mononuclear cells. Albumin-treated cells were heated for 30 min in a water bath at 56°C immediately before they were administered to newborn animals. The percentage of cells which took up trypan blue stain increased from less than 10% before heating to more than 90% after heating while the percentage of free HA (i.e., not cell-bound) in the suspending medium, as judged by radioactive counts, increased from less than 10% before heating to 42–51%

after heating. Since the number of cells per unit volume remained the same before and after heating (the difference was well within the limits of counting error), about 40% of the cell-bound albumin seemed to have come off the cells as a consequence of heating. A group of 18 newborn animals was given 8×10^7 such HA-treated heated mononuclear cells in two injections less than 24 and 48 hr after birth. The dose of HA associated with these cells, before heating was $1.9 - 2.4$ μg N. We failed to detect antibody in any of these animals during the observation period of 2 months. In view of the promoting capacity of rabbit erythrocytes (Table II), it seems that the antibody response induced in very young animals depends on the integrity of albumin-treated, nucleated cells.

Having found that attachment of antigen to nucleated cells, and integrity of the cell were essential to promoting capacity, we examined, next, whether nucleated cells possessed this capacity if they were capable of taking up antigen, but were of an origin quite different from that of the cells so far employed.

A group of 11 animals was given, in two injections, 3.4 μg N HA associated with 8×10^7 L cells less than 24 and 48 hr after birth. None of them formed any detectable antibody during the observation period of 2 months. Although this is only one instance, it may be concluded that the promotion of antibody formation is not a universal property of viable nucleated cells. Consequently, the role that the antigen-treated cells played in the induction of the antibody response might be passive but was certainly far from nonspecific.

The Effect of HA, Given in Soluble Form, during the First 15 Days of Life.—The prolonged integrity of the antigen carrier may be a condition of promoting capacity. However, even with rabbit nucleated cells, the possibility cannot be completely excluded that HA was released from the cells very soon after injection, since the latter, being foreign, might have evoked a homograft reaction. If this were the case the antigen might have been released when the recipient animals were still immunologically immature. Homologous cells may evoke a much milder homograft reaction than the L cells, which would lead to comparatively slow destruction of the injected cells, thereby releasing the antigen when animals had become immunologically mature. If this were the case, we should be able to induce antibody response by injecting soluble HA (not attached to cells) at varying times after birth.

Four groups of animals were given a single injection of 2.8 μg N HA on the 4th day, 6th day, 13th day, or 15th day after birth. None of the five animals injected on the 4th day formed antibody. Of the 34 animals injected on the 6th day four (12%) formed antibody with reciprocal titers varying between 20 and 200 and persisting for relatively short periods. One (14%) of the 25 animals injected on the 15th day formed antibody in the 4th wk of life, with a reciprocal titer of 50, which persisted only for 1 wk. The results are summarized in Table V.

We could not evoke a greater antibody response with larger doses of albumin

A group of 24 animals was given in one injection 15 days after birth 2.8 μg N albumin for every 50 g body weight (on the average 10.3 μg N per animal). Only one animal (4%) formed antibody in the 4th wk of life with a reciprocal titer of 50 that persisted only for 1 wk. This is still very much lower than the antibody response of adult animals (35%) to the same dose of HA per unit

TABLE V

*Response of Rabbits to HA in Solution, Injected 4–15 Days after Birth**

Injection		Fraction of animals which produce agglutinating antibody	Elimination of albumin 68 days after birth		
Amount of HA injected	Time of injection after birth		Number of animals tested	Number of animals showing triphasic elimination	Average t of animals showing triphasic elimination‡
(μg N)	days				(hr)
2.8	4	0/5	4	4	244 ± 27 ($P > 0.5$)
	6	4/34§	14	14‖	222 ± 13 ($0.1 < P < 0.2$)
	13	0/3	3	3	255 ± 23 ($P > 0.5$)
	15	2/25¶	9	8	266 ± 12 ($0.2 < P < 0.5$)
10.3	15	1/24**	4	4	205 ± 37 ($0.2 < P < 0.5$)

* Animals were given a single intraperitoneal injection.

‡ The values in brackets refer to comparison with the mean value for t of 26 normal control animals.

§ Of these 34 animals only four formed detectable antibody with a reciprocal titer of 20–200. In three of them antibody was detected only once. In one animal antibody was detected 20 and 27 days, respectively, after birth.

‖ Only one animal produced agglutinating antibody; the value for t of this animal was only 195 hr.

¶ Two of these 25 animals formed antibody, which was detected only once on the 28th and 35th day of life, respectively, with a reciprocal titer of 50 and 200.

** Antibody was detected only in one of these 24 animals on the 28th day of life with a reciprocal titer of 100.

body weight. Thus, whether 15-day-old animals were injected with 2.8 μg N or with 10.3 μg N HA, the percentage of responders remained the same (i.e., 2/25 and 1/24, respectively, $0.9 < P$). It thus appeared that the responsiveness of young animals to HA (2.8 μg N per 50 g body weight) changed very little during the first 15 days of life.

We next examined the immune status of the animals injected with albumin 4–15 days after birth. Four animals injected with 2.8 μg N albumin on the 4th day, 14 animals injected on the 6th day, 3 animals injected on the 13th day,

and 9 animals injected on the 15th day, and 4 animals injected with 10.3 μg N HA on the 15th day, were given intravenous injections of HA.^{131}I 68 days after birth. One animal injected with 2.8 μg N HA on the 15th day showed diphasic elimination; all the other animals showed triphasic immune elimination. There was no evidence of differences among the five average values for t ($F = 0.63$, $F_{0.01} = 4.07$)[2] none of which was significantly different from the average value for t of 26 normal animals which were not injected at birth (Table II). Thus soluble antigen, given between 0–15 days after birth, does not prime the immune apparatus so as to lead to a secondary response after renewed exposure to antigen in adult life.

2-Mercaptoethanol (ME) Sensitivity of Antibody.—We examined next the ME sensitivity of antibody induced by HA associated with different types of cells and the resulting data are shown in Table VI.

It seemed that there was a correspondence between the "promoting capacity" of cells and the quality of antibody response. HA, attached to erythrocytes, induced a comparatively small fraction of injected animals to form antibody which appeared very late and was in its entirety sensitive to mercaptoethanol treatment. Albumin, taken up by mononuclear cells, induced a much greater fraction of injected animals to form antibody which appeared early and was only initially sensitive to ME treatment. Most of the animals injected with HA, taken up by thymus cells, formed only antibody that was resistant to ME treatment.

In this connection it is of interest that a small quantity of antigen (polio virus) induces only ME-sensitive antibody while a large quantity induces a mixture of ME-sensitive and ME-resistant antibody (25). Thus the qualitative difference between antibody induced by HA covalently linked to erythrocytes and that induced by HA, taken up by nucleated cells, might be attributable to a dose effect. With HA covalently linked to erythrocytes only a small proportion of the antigen, and with HA in association with nucleated cells a relatively large proportion of the antigen might be able to reach target cells. Whether this is more than a superficial analogy must be left to future experimental work.

We have thus found that a quantity of antigen which could induce tolerance in a proportion of animals when injected in soluble form would induce an antibody response when in association with cells and that the incidence of the antibody formation and the time and quantity with which antibody appeared depended on the type of cells with which the antigen was associated. We turned next to the question whether the antibody, induced by HA associated with nucleated cells, was synthesized by donor or by recipient cells.

The Cellular Origin of the Antibody.—The transfer of cell-associated antigen has been found to induce antibody in immunologically incompetent animals (26–31,)

[2] F is the variance ratio and $F_{00.1}$ is the 1% value of F (24).

TABLE VI

*The Agglutinating Capacity of Antibody in the Presence and Absence of 2-Mercaptoethanol (ME)**

Animals injected at birth with:	Reciprocal titers of individual responders on days after birth:					
	14 days	20 days	29 days	35 days	45 days	55 days
HA + Erythrocytes	<10/500	<10/20	<10	<10	<10	<10
	<10	<10	<10	<10	<10/200	<10/200
	<10	<10	<10	<10	<10/50	<10/100
	<10	<10	<10	<10	<10/20	<10/10
	<10	<10	<10	<10	<10	<10/100
	<10	<10	<10	<10	<10	<10/10
HA + Monuclear peritoneal exudate cells	n.d.‡/5000	500/2000	1000/1000	1000/1000	1000/2000	1000/1000
	<10	100/100	<10	<10	<10	<10
	<10	<10	<10	<10	20/200	500/500
	<10	<10	<10	<10	<10/200	100/200
HA + Lymph node cells	<10/50	20/100	<10	<10	<10	<10
	<10	<10/50	<10	<10	<10	10/20
	<10	<10	<10/20	<10	200/200	500/500
	<10	<10	<10/100	<10/20	<10/50	<10
	<10	50/200	<10/20	<10	<10	<10
	<10	<10	<10	<10	<10	10/50
	<10	100/200	<10/100	<10/100	<10/100	1000/2000
	<10	20/50	10/50	50/100	100/100	200/200
	<10	100/100	<10	<10	<10/100	<10/20
	<10	50/50	<10	<10	<10	<10
HA + Thymus cells	<10	<10/100	<10	20/50	20/50	20/50
	<10	<10/100	<10	<10	20/20	20/20
	<10	<10	<10/50	<10	<10	<10
	<10	100/100	50/50	<10	<10	20/50
	<10	100/100	20/20	<10	<10	<10
	<10	<10	100/100	200/200	200/200	200/200
	<10	<10	<10	50/100	100/100	500/500
	<10	<10	<10	20/50	50/50	100/100
	<10	<10	<10	10/10	20/20	500/500
	<10	<10	<10	<10	50/50	100/100
HA + Spleen cells	<10/500	500/1000	500/1000	200/200	200/200	n.d.‡/200
	<10	<10	20/100	<10	10/100	200/200
	<10	<10	<10	<10	20/100	200/200
HA + Pulmonary alveolar mononuclear cells	<10	20/50	20/50	<10/20	<10/100	<10

* Rabbits were injected at birth with HA attached to different kinds of cells. The reciprocal agglutinating titers are shown as fractions. The numerator is the reciprocal titer in the presence of ME; the denominator is the reciprocal titer in the absence of ME. A single value is shown when antibody could not be detected even in the absence of ME.

‡ n.d., not done.

and most investigators have assumed that the antibody was formed by the transferred cells. This view has found experimental support by some (32) but not by other investigators (33, 34). We proceeded to examine this problem.

Animals, used as recipients and donors, differed in the allotypic specificity of

their immunoglobulins and possessed either allotypic specificity A4 or A5. These specificities are known to be located on the light chains and to be determined by two allelic genes A_b^4 and A_b^5 (35). In the majority of animals (72/86) which had been injected with antigen, in association with nucleated cells, we could detect, on the 14th day of life, allotypic specificity of donor origin. The variation with time in the concentration of the allotypic specificity appeared, however, to be quite independent of the variation with time of the antibody to HA (Fig. 4, *I*). It was thus clear that a high proportion of the donor immunoglobulin did *not* have the capacity to combine with HA. To test whether any of the antibody to HA was of donor origin, the antibody was separated by incubation with "immunoabsorbent" (HA, covalently linked to cellulose particles; 23).[1] After incubation, the cellulose particles were separated from the serum by centrifugation, were washed, and antibody was eluted at pH 3.0. The eluate, adjusted to pH 7.4, was then tested for antibody activity and for the presence of allotypic specificities of donor and recipient types.

The quantity of allotypically defined immunoglobulins in isolated antibody or in whole serum was estimated by two techniques: by hemagglutination inhibition (18) and by single diffusion (20). The first of these techniques is capable of detecting very small relative quantities of allotypically marked immunoglobulin, but with a low level of precision. The second method gives relative immunoglobulin quantities with an error of 5–10%, but is much less sensitive than the first. Where both methods could be used, the agreement between relative values obtained by these two methods was good.

The quantity of antibody recovered from the immunoabsorbent was generally between 25% and 100% of the total antibody, but for serum from 2 to 3 wk old animals was usually lower: between 5% and 20%.

The antibody to human albumin isolated from animals injected at birth always contained allotypic specificity of the recipients and never the allotype of the donor of the transferred cells (Fig. 4, *II*).

It seems reasonable to assume that the recovery of antibody of donor and recipient origin would be similar and that low recoveries of early antibody will therefore not affect the validity of our conclusions. Thus, we conclude that transferred cells did not synthesize an important fraction of the antibody. We also examined the supernatants from the sera which had been treated with immunoabsorbent (all detectable antibody to HA was removed by this treatment) and found that the concentration of allotypic specificity of donor type was not affected by the exposure to immunoabsorbent (Fig. 4, *III*). It follows that the remarkable difference between the response of newborn animals to the same antigen in soluble form and in association with nucleated cells must be attributed to the potentiation of the immunogenicity of the antigen and presumably to some kind of mediation between antigen and the synthetic component of the immune apparatus of the newborn animal.

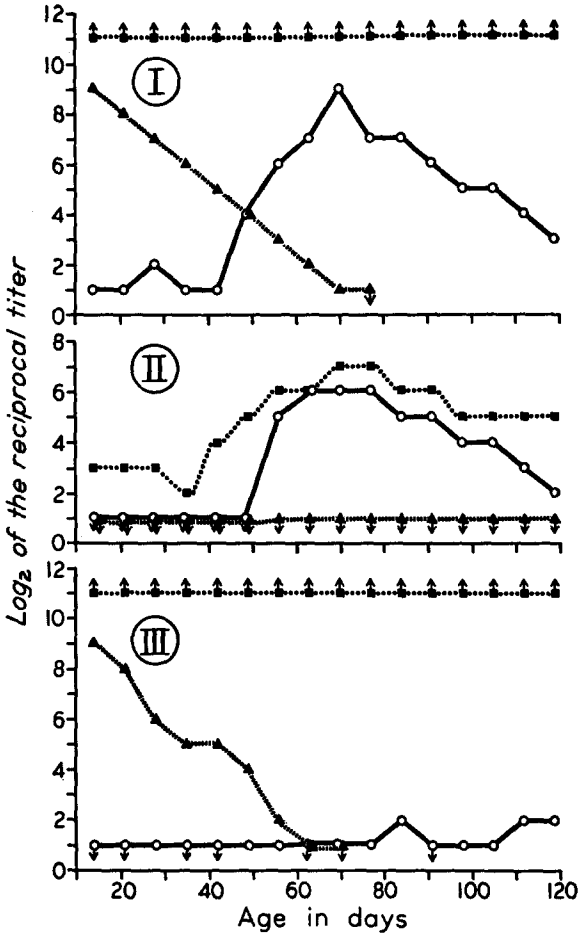

FIG. 4. Examination of the cellular origin of the antibody to HA detectable in the serum of a rabbit (No. 2115B) injected with HA, taken up by mononuclear peritoneal exudate cells. *I*, Titers of antibody to HA and allotypic specificities in the serum. *II*, Titers of antibody to HA and allotypic specificities in the eluate obtained by absorption of serum on cellulose conjugated with HA and elution at pH 3.0. *III*, Titers of antibody to HA and allotypic specificities in the supernatant after absorption with cellulose conjugated with HA. ···■···, Reciprocal titer of recipient—type allotypic specificity. ----▲----, Reciprocal titer of donor-type allotypic specificity. —○—, Reciprocal titer of antibody to HA.

The Cellular Origin of Immunoglobulin.—We have seen that antibodies to HA, found in very young rabbits, were the product of recipient cells. However, we have also detected immunoglobulins which were the product of donor cells (Fig. 4, *I*). This allotypic specificity of donor type could be observed in most

recipients for a period of 4–10 wk, with a continuous decline in concentration until it was no longer detectable. The half-life of disappearance, measured by agglutination inhibition, was about 7 days (not corrected for increasing body weight, Fig. 4, *I*), a half-life quite similar to that found when immunoglobulins were passively transferred (33, 36). The frequency with which allogeneic immunoglobulin could be detected and the quantity of donor allotypic specificity, in animals, injected with different cell types, showed remarkable and significant differences. In 14-day-old rabbits, donor allotype was found in 12/25 animals which had received thymus cells and in 26/26 and 34/35 animals which had been given lymph node cells and mononuclear peritoneal exudate cells, respectively. There was very little donor immunoglobulin if thymus cells had been administered and much more if mononuclear peritoneal exudate cells or lymph node cells had been given (Fig. 5). It thus appeared as if mononuclear peritoneal exudate cells and lymph node cells could produce molecules with allotypic specificity quite soon after injection into the animal, but that this synthetic capacity was being lost after less than 2 wk residence of the donor cells in the recipient.

Chimerism.—The continuous decline of allotypic specificity of donor type, so far described, was observed in most, but not in all recipients. In some animals there was a rapid increase of donor allotypic specificity which continued up to the 45th to 60th day of life and finally resulted in a stabilized concentration of the "foreign" allotype which was quite similar to that found in normal heterozygous animals (Fig. 6). The stabilized level was maintained throughout the period of observation. This active synthesis of donor immunoglobulins was most frequently observed in animals which had received thymus cells (Fig. 5), occasionally in animals which had received lymph node cells (Fig. 5), but never in animals which had received mononuclear peritoneal exudate cells. In the case of animals which had received thymus cells, the stabilized concentration was close to that of untreated heterozygotes (A^4/A^5) and was 10–20% of this value in animals which had been injected with lymph node cells. The increase in foreign immunoglobulin occurred later in animals which had received lymph node cells and was preceded by a phase in which the donor allotypic specificity decreased (Fig. 6).

In order to examine the location of donor cells in animals which, though genotypically A^4/A^4, have acquired the capacity to produce immunoglobulins of allotype A5, we transplanted cells from such animals to newborn rabbits of allotype A^4/A^4. The acquired allotypic specificity (A5) could be detected in the recipients when lymph node cells or spleen cells were transferred but not when thymus cells or bone marrow cells were transferred (Table VII).

DISCUSSION

All the rabbit cells which we used as carriers of albumin showed promoting capacity in inducing very young rabbits to form antibody. It appears that the

decision between antibody response and tolerance induction depends on size rather than on the structure of the antigen. The size may affect the period within the body during which an antigen may retain its configuration and may also affect the rate at which it is phagocytized. Whether such phagocytosis is itself an active step in the initiation of the immune response (37, 38) or whether

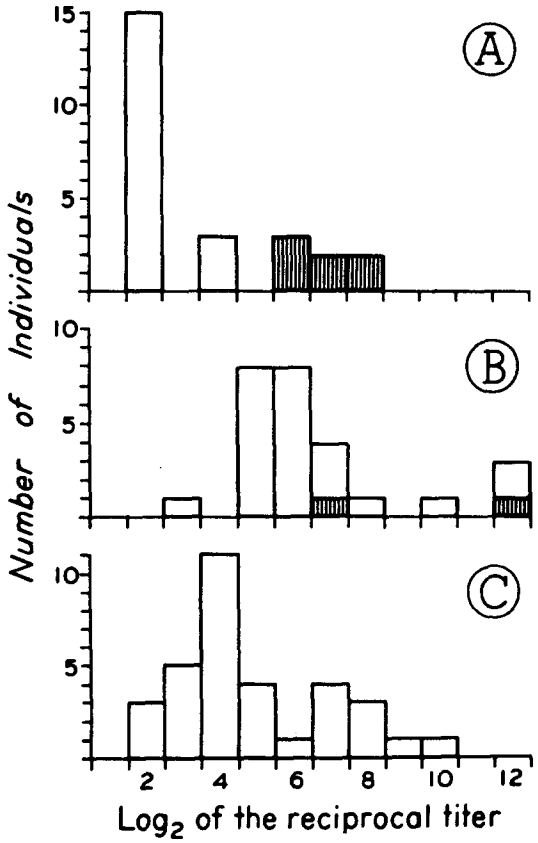

FIG. 5. Presence of donor-type allotypic specificity in the sera of 14-day-old rabbits. *A*, Rabbits injected with thymus cells. *B*, Rabbits injected with lymph node cells. *C*, Rabbits injected with mononuclear peritoneal exudate cells. Hatched areas represent individuals in which the donor-type allotypic specificity was present throughout life (chimeras).

it simply serves to reduce the ambient antigen concentration in the immediate surroundings of antibody-forming cells must remain open. Promoting capacity of cells does not solely depend on size of the antigen: the promoting capacity of red blood corpuscles and nucleated cells shows considerable differences. These may be attributable to processing of antigen by nucleated cells and to superior ability of the nucleated cells to reach antibody-forming organs. By these

processes the antigen may be prepared and then presented to antibody-forming cells. While there is no conclusive evidence that the processing of the antigen does occur, it may be concluded from the secondary transfer experiments (Table VII) that the nucleated cells reach various antibody-forming organs.

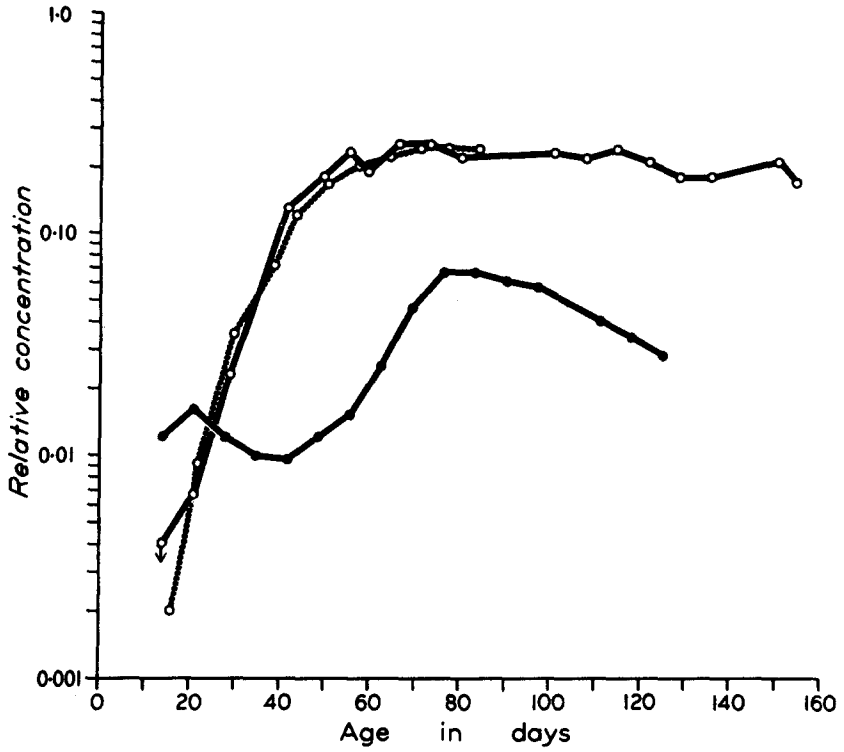

FIG. 6. Concentration of donor allotypic protein (immunoglobulin) in the serum of rabbits injected at birth with nucleated cells. Comparison between concentration of allotypic immunoglobulin in chimeric animals and in normal heterozygotes. Allotype of recipient animals was A5 and allotype of donor animals was A4. Allotype concentration was measured by a single diffusion method. —○—, Concentration of allotypic protein of donor type in a rabbit injected at birth with thymus cells. —●—, Concentration of allotypic protein of donor type in a rabbit injected at birth with lymph node cells. ---○---, Concentration of allotypic immunoglobulin in normal heterozygous animal.

The capacity of nucleated cells to promote antibody formation in very young animals is distinct from their capacity to synthesize or to induce synthesis of serum proteins which carry the marker of the gene product of the transferred allogeneic cells. Indeed, it seems highly probable that different cell types or at least cells at different stages of differentiation are responsible for these two functions.

We have seen that relatively high concentrations of donor immunoglobulins are found in the recipient's circulation within 2 wk after transfer and that active synthesis of allogeneic allotypic specificity has at this time ceased in all animals which received mononuclear peritoneal exudate cells and in most animals which received lymph node cells. It is likely that cells in these populations are releasing immunoglobulins soon after they have been transferred. Alternatively, information transfer from the donor cells might have enabled recipient antibody-forming cells to produce immunoglobulins carrying the allotypic marker of the donor (39, 40).

The concentration of foreign allotype in the serum of some animals which received thymus cells and of a few which received lymph node cells showed a

TABLE VII

*Secondary Transfer**

Donor cells	Presence of A5 in the recipient‡ Cells incubated with		Presence of antibody to HA§
	HA	—	
Thymus cells	0/4	0/5	3/4
Lymph node cells	7/10	3/3	9/9
Spleen cells	3/3	—	2/2
Bone marrow cells	0/3	0/4	1/2

* Newborn rabbits of allotype A^4/A^4 were injected with cells from adult donors. The donors were genotypically A^4/A^4 but had acquired the capacity to produce immunoglobulins of allotype A5 as a consequence of neonatal transfer.

‡ On the 14th day of life.

§ In animals who received donor cells at birth which had been incubated with HA.^{131}I in vitro. Animals were considered as responders if antibody had been formed during the first 35 days of life.

steady increase during the initial period of observation and reached stable levels during the rest of the observation period. Clearly, active synthesis of donor allotype was taking place in these animals.

It thus seems reasonable to assume that chimerism (41–61) has been produced within a noninbred animal population by the transfer of cells to newborn animals. Active synthesis of allogeneic immunoglobulin may be attributed to descendants of stem cells which formed a proportion of the transferred cells. Differences in the rate and final concentration of allogeneic immunoglobulin may reflect differences in the number and properties of cells at various stages of differentiation.

The chimeric animals, which resulted from the transfer of thymus cells, produced allogeneic allotype at a rate and at concentrations comparable to those

found in heterozygous animals. The transfer of lymph node cells led to a later appearance of immunoglobulin, to an increase at a slower rate and to a markedly lower level of stabilized production. This difference in the kinetics of immunoglobulin production may be interpreted in terms of a relatively large number of stem cells in the thymus and of a relatively small number of stem cells in the lymph node. Donor stem cells would compete with recipient stem cells for the space available for antibody-forming cells. The thymus would contain stem cells in sufficient numbers to compete with recipient cells for cell space in a manner which corresponds to that normally found in heterozygous animals. The lower initial number of stem cells in the lymph node would finally result in a smaller share of the total cell space. In short, cell populations derived from different sites differed markedly in their capacity to induce long-lasting appearance of allogeneic immunoglobulin in the recipient. It would appear that there is a high proportion of cells in the thymus which can permanently colonize the recipient and which can go on to differentiate to a point where allotype is being formed, and that there is a much smaller proportion of such cells in the lymph node. Peritoneal exudates seem to be deficient in such stem cells. While chimerism depends on undifferentiated cells, the appearance of allogeneic immunoglobulin, shortly after cell transfer ($\ll$14 days) depends on more highly differentiated cells. The concentration of such cells in different populations would be expected to be highest when the stem cell content is lowest. Indeed the more highly differentiated cells are more prevalent in mononuclear peritoneal exudates and among lymph node cells and are scarce among thymus cells. Lymph node cells are thus intermediate between thymus and mononuclear peritoneal exudate cells in the relative proportion of undifferentiated stem cells and differentiated immunoglobulin-producing cells.

The diphasic appearance of the "foreign" immunoglobulin (Fig. 6) in lymph node cell-induced chimeras can also be interpreted in terms of the above considerations. It seems quite possible that, during the first phase, immunoglobulin was being produced by cells which were already fairly far advanced in their differentiation at the time of transfer and that the decline of immunoglobulin concentration reflected the life span of these differentiated cells. The second, long-lasting rise might be attributable to the synthetic activity of cells, much less differentiated at the time of transfer. Products from these cells would constitute an appreciable fraction of the immunoglobulins only after cell differentiation and cell division had progressed sufficiently.

SUMMARY

The injection into newborn rabbits of a small quantity of human albumin, associated with red blood corpuscles or nucleated rabbit cells, induces an antibody response in the majority of animals, whereas the same quantity of antigen

in solution fails to stimulate antibody formation or induces tolerance. The promoting capacity of the cells depends on attachment of antigen to them.

The antibody produced after the injection of albumin, associated with nucleated cells, is of recipient origin. However, immunoglobulin carrying the marker of donor cells can be demonstrated in the recipient animals, and may reach serum concentrations similar to those normally present in animals which are heterozygous with respect to the marker. It appears that the antibody-promoting function and the synthetic capacity for allotype are quite distinct and that the period required for allotype formation is very short with mononuclear peritoneal exudate cells and is very much longer with cells from the thymus. The capacity of cells from lymph nodes for sustained allotype formation is less than that of thymus cells but greater than that of mononuclear peritoneal exudate cells.

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