THE NATURE OF THE IMMUNOLOGIC INADEQUACY OF NEONATAL RABBITS AS REVEALED BY CELL TRANSFER STUDIES*,‡

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It is generally accepted that the immunologic mechanisms of the neonatal animal are poorly developed and in some instances virtually non-existent. This immunologic inadequacy early in life has been related both to the limited ability of the neonatal animal to synthesize gamma globulin and to the immunologic inexperience of the organism. However, it has not been determined whether the underlying inadequacy is a lack of cells capable of antibody synthesis for whatever reason, or an environment within the animal incompatible with the immunologic function of potentially capable cells.

The technique of transferring cells capable of an antibody response to recipient animals offered a possible approach to the study of the immunologic inadequacy of the neonatal animal. Either primary or secondary antibody responses have been elicited in adult x-radiated or normal homologous recipients (1) after the transfer of cells appropriately stimulated with antigen. It seems likely, on the basis of considerable immunologic evidence, that in these transfer experiments it is the transferred cells that are primarily responsible for the observed antibody responses and that the adult recipients play a supporting or secondary role. If this is true, the performance of immunologically competent cells after their transfer to neonatal recipients should throw light on the nature of the inadequacy of the neonatal animal. If the inadequacy of the neonatal animal were merely the lack of immunologically competent cells, properly stimulated cells transferred to neonatal should make an antibody response, especially since the neonatal recipients would not be likely to react strongly to the transferred cells. On the other hand, if something needed to support immunologically active cells were lacking in the internal environment of the neonatal animals, or if these immature animals possessed a factor capable of interfering with immunologic processes, properly stimulated cells trans-

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ferred to them would be unable to make an antibody response. The findings of the present study suggest that the immunologic inadequacy of the neonatal animal is related to its internal environment and not necessarily to the lack of cells capable of antibody synthesis.

Materials and Methods

1. Antigens used included: Bovine serum albumin, crystallized (BSA), Pentex Corp., New York, Lot No. B-12016 P; Bovine gamma globulin (BGG), Armour and Co., Chicago, Lot No. 128213; and a soluble extract of *Shigella paradysenteriae* (ST) prepared by digestion of the organisms with trypsin as described by Harris *et al.* (2).[1]

2. Albino donor rabbits weighing approximately 2.5 kg. were used as sources of cells in all experiments. In experiments 201 and 205, Table I, the donors were normal, non-immunized. In experiments 207, 209, and 218, Table I, the donors had been previously immunized by a series of injections of BSA totalling 230 mg., the last injection of which was given approximately 3 weeks prior to the cell transfer (1 M). In all these experiments employing serum protein antigens, mesenteric and popliteal nodes were used for transfer. In experiments 211, 213, 217, and 221, Table II, the donors had been given 0.2 μg. of ST into the pad of each foot and popliteal and axillary nodes were used for transfers. For experiments 211 and 213 the nodes were obtained 2 hours after injection of ST, for experiment 217 they were obtained three days after injection of ST, and for experiment 221 they were obtained 1 day and 3 days after injection of ST. The lymph node cell suspensions were prepared, counted, and injected subcutaneously and intramuscularly as previously described into all adult and immature recipients except the adults in experiment 211 in which the intravenous route was used (1 M).

3. Recipient rabbits were either 5 to 6 days old or adult albinos weighing 2.5 kg. As indicated in Tables I and II the adult recipients were either untreated normals or x-radiated (400 r whole body x-radiation 2 days before injection of cells). The recipients of experiments 201, 205, 207, 209, and 218, Table I, received I^{131}-labelled antigen, I*BSA or I*BGG prepared as previously described (3), either intramuscularly a few hours before the transfer of cells in the case of the immature rabbits or intravenously at the time of transfer of cells in the case of the adults. Sera for antibody determinations were obtained from adult recipients 3 or 4 days after elimination of antigen and from neonatal recipients by cardiac puncture at various times between 7 to 14 days after transfer of cells. The methods of antigen and antibody determination used with BSA and BGG have been previously described (1 *m*). In experiments 211, 213, 217, and 221, Table II, the recipients received cells recently challenged with ST and no additional antigen. The adult recipients were bled daily for serum agglutinin determinations while the neonatal recipients were bled by cardiac puncture every 2 to 3 days. The determinations of agglutinins titres for *Shigella paradysenteriae* were made according to the method described by Harris *et al.* (1 *g*).

Sites of injection of the lymph node cells from both the neonatal and adult recipients above were taken for histologic study at the time of the last bleeding. In addition, other neonatal and adult recipients were sacrificed from 1 to 8 days after transfer and the injection sites were prepared for histologic study in order to provide a relatively complete picture of the morphologic development of the transferred cells and of the host response.

Procedures.—In the experiments using serum protein antigens we attempted to elicit a primary response (Experiments 201 and 205) from normal adult lymph node cells transferred

[1] The ST was kindly supplied by Dr. T. N. Harris of the Children's Hospital of Philadelphia.

TABLE I

Antibody Responses to Serum Protein Antigens by Lymph Node Cells Transferred to Neonatal and Adult Recipients

Exp. No.	Source of lymph node cells	No. cells/ recipient	Recipients	No. recipi- ents	Antigen/recipients	Day of elimination of antigen‡				Average μg. Ab N/ml. Serum§
						5	6	7	8	
201	Mesenteric and popliteal nodes of normal adult rabbits	4.7×10^8	5 day old	6	0.9 mg. I*BSA-IM 5 × ½ hrs. before transfer	—	0/6	—	0/6‖	0
		0	5 " "	2	0.9 mg. I*BSA-IM	—	0/3	—	0/3‖	0
205	Mesenteric and popliteal nodes of normal adult rabbits	4.0×10^8	6 day old	7	0.9 mg. I*BGG-IM at time of transfer	—	0/7	—	0/7¶	0
		0	6 " "	3	0.9 mg. I*BGG-IM	—	0/3	—	0/3¶	0
207	Mesenteric and popliteal nodes of adults previously immunized with BSA	2.0×10^8	5 day old	12	0.6 mg. I*BSA-IM 4 hrs. before transfer	0/12	—	0/12	—	0
		5.0×10^8	Normal adult	5	1.5 mg. I*BSA-IV at time of transfer	1/5	3/5	4/5	5/5	1.1
		5.0×10^8	400 r x-ray adults	7	1.5 mg. I*BSA-IV at time of transfer	6/7	7/7	—	—	2.2
209	Mesenteric and popliteal nodes adults previously immunized with BSA	2.0×10^8	5 day old	5	0.3 mg. I*BSA-IM 4 hrs. before transfer	0/5	—	0/5	—	0
		2.0×10^8	5 " "	5	0.03 mg. I*BSA-IM 4 hrs. before transfer	0/5	—	0/5	—	0
		5.0×10^8	400 r x-ray adults	8	1.5 mg. I*BSA-IV at time of transfer	4/8	6/8	7/8	—	6.6
218	Mesenteric and popliteal nodes of adults previously immunized with BSA	2.0×10^8	6 day old	4	0.3 mg. I*BSA-IM 4 hrs. before transfer	0/4	—	0/4	—	0
		2.0×10^8	6 " "	4	0.06 mg. I*BSA-IM 4 hrs. before transfer	0/4	—	0/4	—	0
		5.0×10^8	Normal adults	5	1.5 mg. I*BSA-IV at time of transfer	2/5	5/5	—	—	1.1
		5.0×10^8	400 r x-ray adults	5	1.5 mg. I*BSA-IV at time of transfer	5/5	—	—	—	12.1

‡ Numerator is number animals having eliminated antigen from circulation and denominator is total number of animals in group.

§ μg. Ab N obtained by multiplying μg. antigen N precipitated by 1 ml. of serum at 80 per cent antigen precipitated by the average antibody N/antigen N ratio at this point (5.5).

‖ Antigen still circulating day 14 in all recipients.

¶ Antigen still circulating day 11 in all recipients.

TABLE II

Antibody Responses to Bacterial Antigens by Lymph Node Cells Transferred to Neonatal and Adult Recipients

Exp. No.	Source of lymph node cells	No. cells/ recipient	Recipients	No. recipients	Reciprocal of Maximum agglutinin titres	Time of maximum titre after transfer
						days
211	Popliteal and axillary nodes from donors injected with 0.2 μg. ST in each foot 2 hrs. before transfer	1.0×10^8	5 day old	7	<8, <8, <8, <8, 12, 12, 12	—
		$2.5 \times 10^{8*}$	400 r x-ray adults	2	96, 192	7
213	Popliteal & axillary nodes from donors injected with 0.2 μg. ST in each foot 2 hrs. before transfer	1.0×10^8	6 day old	5	<8, <8, 12, 12, 12	—
		2.5×10^8	400 r x-ray adults	5	48, 96, 384, 384, 384	6–8
217	Popliteal and axillary nodes from donors injected with 0.2 μg. ST in each foot 3 days before transfer	1.0×10^8	5 day old	4	192, 192, 192, 1536	4–6
		2.5×10^8	400 r x-ray adults	5	96, 96, 192, 192, 384	4–6
	Popliteal and axillary nodes from donors injected with 0.2 μg. ST in each foot 1 day before transfer	0.56×10^8	5 day old	4	<8, <8, <8, <8	—
221	Popliteal and axillary nodes from donors injected with 0.2 μg. ST in each foot 3 days before transfer	0.5×10^8	5 day old	5	48, 96, 96, 192, 192	4
		2.5×10^8	Normal adult	4	96, 96, 96, 192	4–5

* Cells injected intravenously—all other injections of cells in these experiments were made subcutaneously and intramuscularly.

to neonatal recipients previously injected with antigen, and a secondary response (Experiments 207, 209 and 218) from lymph node cells of previously immunized donors transferred to neonatal recipients previously injected with antigen. The secondary response experiments included as controls the transfer of similar cells to adult recipients either normal or x-radiated. No adult recipients were used in the primary response experiments since it had already been demonstrated that with these antigens a primary response could not be obtained from normal cells transferred to x-radiated adults (1 *m*). The details of these experiments including numbers of cells transferred and amounts of antigen used are given in the first 6 columns of Table I. The number of cells transferred and the amount of antigen injected were based on findings in earlier studies (1 *m*). A wide range of antigen doses was used in the neonatal recipients in order to rule out the possibility that the negative observations were the results of either too much or too little antigen. The larger doses of antigen used in the neonatal recipients approached the antigen-transferred cell ratio used in the adult recipients. The smaller doses of antigens for the neonatal recipients in experiments 209 and 218 were used in an attempt to compensate for the smaller size of these recipients so that the concentrations of antigen in their tissues would approach the concentrations found in adult recipients.

In experiments using the *Shigella* antigen we attempted to obtain agglutinin responses from lymph node cells stimulated by antigen either 2 hours, 1 day, or 3 days prior to their transfer to neonatal recipients. Controls consisted of the transfer of similar cells to adult x-radiated or normal recipients. The details of these experiments are given in the first 5 columns of Table II. The number of cells transferred and the dose of antigen have been based on earlier studies by Harris *et al.* (1 *g* and 1 *l*).

RESULTS

In the last two columns of Table 1 are listed the observations of antigen elimination and maximum serum antibody concentration for the serum protein antigen experiments. The determination of antigen elimination provides the earliest and most sensitive measure of response to these protein antigens (3). In none of the neonatal recipients of either normal or previously immunized cells was there any evidence of an immune response. In the primary response experiments, antigen was found circulating in the neonatal recipients for 14 days in experiment 201 and for 11 days in 205, a much longer time than is usually necessary for elimination of antigen in the primary response in adult rabbits. In the experiments with cells from previously immunized donors, antigen was still circulating in all neonatal recipients 7 days after transfer, while it had been completely eliminated from the circulation of 26 of 30 adult recipients of the same cell suspensions within 6 days. Previous experience with adult recipients indicated that if antigen was not eliminated within 6 or 7 days there would be no significant response. While the serum antibody levels achieved in the adult recipients were not particularly high, all groups did show some antibody. In the neonatal recipients no antibody could be detected in sera obtained from 7 to 14 days after tranfer of cells. Reducing the dose of antigen in the neonatal recipients as was done in experiments 209 and 218, so that the environment of the transferred cells would have a concentration of antigen similar to that in adult recipients, did not improve the responses in neonatal recipients. With the wide range of antigen-transferred cell ratios

employed, the failure of the responses in neonatal recipients was most likely not the result of the dose of antigen.

The maximum agglutinin titres achieved by the neonatal and adult recipients of the ST-stimulated cells are given in column 6 of Table II and the time of the maximum titre in days after transfer is given in column 7. In both experiments 211 and 213 in which the cells were taken from the donors 2 hours after the injection of ST, there was no significant development of antibody in the neonatal recipients, while the adult recipients showed considerable agglutinin titres. The titres of 12 shown by some of the neonatal recipients are not considered significant since the sera of non-immunized control rabbits frequently demonstrate this degree of reaction. In addition, these low titres were found regardless of when the bleedings were made, suggesting that they were probably related to passively transferred maternal antibody and not to a response in the neonatal recipient. The interval of 6 to 8 days for the development of peak titre after transfer of recently stimulated cells to x-radiated adults is in agreement with previously published observations (4). In experiments 217 and 221, in which cells were transferred 3 days after stimulation by ST, the neonatal and adult recipients developed comparable, significant agglutinin titres 4 to 6 days after transfer. However, in experiment 221 the transfer of cells to neonatal recipients 1 day after injection of ST in the donors did not result in a detectable response. The shorter interval between transfer of cells 3 days after stimulation and peak titre in these experiments is in keeping with the longer interval between injection of antigen into the donor and transfer of cells.

Histologic study of the cell transfer sites in neonatal and adult recipients indicated that the transferred cells seemed to survive in comparable numbers in both kinds of recipients, but in the neonatal recipients plasma cells did not develop in the transfer sites as they did in the adult recipients. A histologic account of the development of the sites of transfer of lymph node cells in adult recipients has been reported earlier (5). The essential histologic features in the adult recipients included:

1. Within the first 3 days many of the transferred cells in the centers of the deposits died while those at the periphery survived and tended in part to cluster about the nerves and vessels in the adjacent subcutaneous and muscular tissue. By the 2nd or 3rd day in the periphery of the injected cell deposits there were found large, primitive appearing cells with amphophilic or basophilic cytoplasm similar to the transitional and preplasma cells of Fagraeus (6).

2. From 4 to 5 days after transfer typical plasma cells began to develop in these transfer sites.

3. From 6 to 8 or 9 days after transfer the plasma cells were prominent and were found roughly in proportion to the size of the associated antibody response.

4. The adult host response consisted of an early polymorphonuclear reaction especially about the dead cells and then of a fibroblast and giant cell response which was first evident about 3 to 4 days after transfer and became progressively greater.

In the neonatal recipients the lymph node cells, transferred either prior to antigenic stimulation or 2 hours after stimulation, scattered somewhat more evenly in the subcutaneous tissues than they did in adult recipients. Within the first 3 days there was death of cells principally in the centers of the injection sites, while the cells at the periphery were well preserved. At this time the lymphocytes did not show the tendency to accumulate about nerves and vessels as they did in the adults. On the 2nd or 3rd day after transfer the injection sites contained few if any of the large, primitive plasma cell precursors. From 4 to 5 days after transfer the lymphocytes began to aggregate about the nerves and vessels in the subcutaneous tissue and muscle. Most of the cells in the sites were lymphocytes and a relatively small number were large basophilic cells or plasma cells. There were still sparsely scattered lymphocytes throughout the injection sites, something rarely seen in adult recipient. From 6 to 8 days after transfer the picture changed little from that seen at 4 to 5 days. The development of perineural and perivascular lymphoid aggregates continued, but the number of preplasma cells and plasma cells in the aggregates never became large. The response of the neonatal recipients consisted of a polymorphonuclear exudate within the first few days and then a diffuse histiocytic reaction throughout the injection site from the 2nd or 3rd day on. Little fibroblastic or giant cell reaction was evident and the debris from necrotic transferred cells was incompletely removed during the 8 day observation period. Limited observations of the sites of cells transferred to neonatal recipients 3 days after injection of antigen (Experiments 217 and 221) showed more plasma cells and plasma cell precursors 4 to 6 days after transfer than were found with cells transferred 2 hours after injection of antigen.

DISCUSSION

Interpretation of the present observations depends in large part on whether the antibody responses found by numerous observers after the transfer of sensitized cells to adult recipients are primarily caused by the transferred cells themselves or whether the recipient also participates directly. There is much to suggest that the transferred cells are primarily responsible and that the recipient merely plays a supporting role.

First, radiation of the recipient prior to the transfer of cells improves the subsequent antibody responses, presumably by preventing the recipient from rejecting the transferred cells (7). This suggests that the recipient is not participating directly in the antibody response, for if he were, x-radiation would be expected to reduce the response. Second, in those experiments in which the cells were stimulated in the donor

prior to transfer, the appearance of antibody in the recipient was related to the time of antigenic stimulation in the donor and not to the time of transfer (4). For example, if the transfer of cells was delayed for 3 days after the injection of antigen in the donor, the appearance of antibody in the recipient occurred 3 days earlier than, if the transfer had been made on the day of stimulation. Third, in some of the experiments in which the transferred cells received a secondary stimulation and were then put in previously unexposed recipients, immunochemical analysis of the antibody appearing in the recipients showed that it was characteristic of a secondary response indicating that it was made by the transferred cells and not the recipient (1 *m*).

If, as appears to be the case, the transferred cells are making the observed antibody responses in these cell transfer experiments, it follows from the present observations that the neonatal recipient must be incapable of providing a suitable environment for cells to make an antibody response.

On the other hand, if in successful cell transfer experiments the recipient animal did more than merely provide a suitable environment for the transferred cells and actually participated in the antibody response, the interpretation of the present observations would be different. If, for example, the transferred cells supplied the first step or steps in the response and the recipient the last step, *i.e.* antibody synthesis, the failure of cell transfers to effect antibody responses in neonatal recipients could be explained on the basis of a lack of cells in the neonatal animals capable of antibody synthesis. However, in the face of the available immunologic evidence this alternative does not seem likely.

The environment provided by the neonatal recipient was not adequate to support the immunologic functions of cells capable of either a primary or a secondary response. The critical period during which the environment provided by neonatal recipients was incapable of adequately supporting the transferred cells was the beginning of the response, lasting somewhere between 1 and 3 days. Cells transferred to neonatal recipients either 2 hours or 1 day after antigenic stimulation failed to make a response while cells transferred 3 days after stimulation by antigen made a good response. Thus, it appears that the activities of the transferred cells during the first few days of an antibody response either were dependent upon factors lacking in the neonatal animal or were interfered with by factors present in the neonatal animal or both. However, if the initial steps in the antibody response were achieved in the donor animal, the actual synthesis and secretion of antibody by transferred cells could proceed in neonatal recipients as indicated by the results of the transfer of cells 3 days after antigenic stimulation.

The exact nature of the unsuitability of the neonatal recipient is not apparent, but it is not likely that it results merely from the failure of the transferred cells to survive or "take." The histologic appearance of the cells after transfer to neonatal recipients and the antibody production by the cells transferred 3 days after antigenic stimulation both indicate that the cells remain

viable after transfer. In addition, it is not likely that neonatal animals with their poorly developed immunologic mechanisms would reject the transferred cells more effectively than would adult recipients.

Not only did the cells transferred to neonatal recipients fail to make an antibody response, but plasma cells also failed to develop in significant numbers in the injection sites. This is in agreement with earlier observations of the parallelism between antibody response and appearance of plasma cells in the transfer sites (1 *m*). The persistence of the transferred lymphocytes without morphologic change in the neonatal transfer sites is in marked contrast to the situation in adult transfer sites where the lymphocytes disappear a few days after transfer and are replaced by plasma cell precursors and mature plasma cells. The failure of plasma cell formation in the neonatal recipient in the presence of the persisting, unchanged, transferred lymphocytes is suggestive evidence that in the adult recipient the plasma cells in the transfer sites arise in large part from the transferred lymphocytes.

Certain comparisons can be made between the immunologic function of cells transferred to neonatal recipients and to *in vitro* cultures. In both situations cells exposed to antigen after transfer, or cells transferred up to 1 day after antigenic stimulation fail to make detectable antibody. On the other hand, cells or tissues transferred 3 days after antigenic stimulation can make considerable amounts of antibody in either neonatal recipients or *in vitro* culture. In view of these similarities between the neonatal recipient and the *in vitro* culture, and since the inability of the *in vitro* culture to support cells in the early part of the antibody response is probably the result of an inadequacy in the environment, it is suggestive that the unsuitability of the neonatal recipient is also related to an inadequacy in the environment it provides rather than to an active interference with immune processes.

A considerable parallel exists between the immunologic potentialities of the neonatal animal and the agammaglobulinemic patient. Neither produces significant amounts of gamma globulin or antibody. Both will serve as adequate recipients for antibody-producing, homologous lymphoid cells. In the agammaglobulinemics, the transfer of lymphoid cells from normal human beings who had been hyperimmunized with typhoid vaccine and diphtheria toxoid resulted in significant production of typhoid agglutinins and diphtheria antitoxin for 2 to 3 months (8). This is probably comparable to the response of lymphoid cells following their transfer to neonatal rabbits 3 days after antigenic stimulation, at a time when antibody synthesis was underway. There is no information as to whether homologous lymphoid cells transferred shortly before or after antigenic stimulation would be unable to carry out the entire response in agammaglobulinemics as in the case of neonatal recipients.

The bases for the immunologic defects of neonatal and x-radiated adult animals would seem to be quite different in the light of the present observations. In both neonatal and x-radiated adult animals there is a similar inability

to initiate immune responses (9) and in both lasting immunologic unresponsiveness can be induced with large amounts of antigen (10). However, in the present experiments the transfer of cells to x-radiated adult animals resulted in good responses while the transfer of similar cells to neonatal animals resulted in no responses. Thus, it would seem that the x-rayed adult animals lack cells competent to make a response while the neonatal animal cannot provide a suitable environment for competent cells if they are supplied.

SUMMARY

Lymph node cells capable of either primary or secondary antibody responses following transfer to adult normal or x-radiated homologous recipients make no response following transfer to neonatal homologous recipients. On the basis of the present observations it seems that the environment provided by the neonatal recipient is unsuitable for the immunologic activities of transferred cells in the early phases of the immune response. Neonatal recipients can, however, adequately support cells transferred during the process of active antibody formation. These findings suggest that the immunologic inadequacy of the neonatal animal is related to its internal environment and not necessarily to the lack of cells capable of antibody synthesis.

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