SURFACE ANTIGENS OF IMMUNOCOMPETENT CELLS

I. Effect of θ and PC.1 Alloantisera on the Ability of Spleen Cells to Transfer Immune Responses*

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Claman et al. (1) made the initial observation that a synergism exists between thymocytes and bone marrow cells in the transferred primary response to sheep erythrocytes (SE)[1] in mice. Subsequently Mitchell and Miller (2) demonstrated that, while thymocytes or thymus-derived thoracic duct cells have an essential role in this response, the actual precursors of the antibody-forming cells are not thymus derived and originate in the bone marrow.

When working with bone marrow and thymus cells it is difficult to analyze whether similar cooperation occurs between these cells in the transfer of immunological memory, as neither cell type is particularly rich in memory cells. In the spleen, where immunological memory can be demonstrated as early as 1 day after injection of SE (3), the descendants of the two populations are mixed and can not readily be separated.

In the present studies an attempt has been made to selectively kill one or the other of these two populations by exposure of spleen cells to specific antisera and complement before cell transfer. Advantage was taken of alloantisera to the θ antigen (4), which is present on thymocytes and thymus-derived peripheral lymphocytes (5–7) and lacking on antibody-forming cells (8), for the specific elimination of the thymus-derived population from spleen cells. In addition, attempts were made to affect the precursors of antibody-forming cells by employing alloantisera to the recently described PC.1 antigen, known to occur on myeloma cells and antibody-forming cells from $PC.1^+$ strains of mice (8).

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[1] *Abbreviations used in this paper:* SE, sheep erythrocytes; NMS, normal mouse serum; PFC, plaque-forming cells; TD, thymus-derived; HA, hemagglutinin.

Materials and Methods

Animals.—BALB/c (H-2^d, θ-C3H, PC.1$^+$) and DBA/2 (H-2^d, θ-C3H, PC.1$^-$) mice were obtained from the colonies maintained in the Sloan-Kettering Institute, New York or from the Jackson Laboratory, Bar Harbor, Maine. In some experiments the donor mice were immunized, 1–4 wk before the day of cell transfer, by intravenous injections of 0.1 ml of 20% SE with or without 10 μg *Escherichia coli* (E. coli) endotoxin (Difco Laboratories, Detroit, Mich.). Recipient mice received 650 R whole body X-irradiation 1 day before cell transfer. Total body irradiation (650 R) was delivered from a Maxitron 300 X-ray machine (half-value layer 2 mm Cu) (General Electric Co., Long Island City, N. Y.).

Antisera.—Alloantisera detecting cell-surface antigens θ-C3H (AKR anti-C3Hf/Bi thymocytes) and PC.1 (DBA/2 anti-BALB/c myeloma MOPC-70A cells) were prepared according to methods previously described (4, 8). Normal mouse serum (NMS) from DBA/2 or BALB/c mice were used as control sera. Viability of cells was determined before and after incubation with antisera by means of the trypan blue exclusion test. After incubation with NMS or anti-PC.1 the degree of cytotoxicity obtained was always smaller than 10%; after anti-θ-C3H there was 25–30% cell death.

Cell Transfer.—Cell suspensions were obtained by teasing spleens or thymuses from donor mice into Medium 199. The cells were filtered through a steel mesh and collected by centrifugation. Cell doses of viable cells needed for groups of 4–5 recipient mice were incubated with NMS or with antiserum and rabbit complement (C′).

The incubation of the cells was performed as follows: To 5–10 × 10^7 cells in 5 ml of Medium 199, held in a 40 ml tube, were added 5 ml of a 1:10 dilution of antiserum or NMS and 5 ml of a 1:15 dilution of fresh rabbit serum (C′) (both diluted in Medium 199) to bring the final cell concentration to 3.4–6.7 × 10^6 cells per ml. Following incubation for 45 min at 37°C, the cells were washed twice and injected intravenously in total doses of 1–2 × 10^7 cells (viable and dead) per recipient. In some cases unincubated thymus cell suspensions (2 × 10^7 cells per recipient) or normal spleen cells (10^7 per recipient) were mixed with the preincubated cells before injection. Approximately 1–2 hr later the recipients were intravenously injected with 0.1 ml of a 20% suspension of SE.

Assay of Immune Response in Recipients.—Recipient mice were exsanguinated on days 4–6 after transfer of a secondary response, or days 6–7 after transfer of a primary response. In some experiments spleens of all individual mice were teased into a suspension and analyzed for the presence of plaque-forming cells (PFC) by the technique of Jerne and Nordin (9). In other experiments spleens of three to five mice were pooled before teasing. IgM or direct PFC were developed by addition of 2 ml of a 1:6 dilution of fresh guinea pig C′, while IgG or indirect PFC were similarly detected after prior incubation with 2 ml of a 1:200 dilution of a rabbit antiserum to mouse immunoglobulin (prepared by immunization with ammonium sulfate-precipitated normal mouse IgG). Hemagglutinin serum titers of individual mice were determined by twofold dilutions in saline.

RESULTS

Transfer of the Primary Response to SE.—Recipients of 2 × 10^7 normal BALB/c spleen cells developed large numbers of direct PFC in their spleens by days 6–7 after transfer and high serum antibody titers (Table I). The numbers of PFC obtained were similar to those reported by others (10). X-irradiated mice receiving antigen alone or antigen with BALB/c thymus cells had insignificant numbers of PFC at this time. Such recipients also had very low, if any, hemagglutinating antibody in their sera (Table I).

Incubation of the donor spleen cells with anti-θ-C3H and C′ reduced the number of PFC in recipient spleens to 17% in one and smaller than 5% in the other two experiments. This inhibition was far greater than the degree of cytotoxicity observed after incubation with anti-θ-C3H and C′, which never exceeded 30% according to the cytotoxic test. The detrimental effect of anti-θ-C3H on the ability of spleen cells to transfer an immune response was also evident from the 5–7 log 2 reduction in serum hemagglutinin titers seen in these recipients. Addition of 2×10^7 thymus cells to the anti-θ-C3H–incubated spleen cells resulted in a reconstitution of the ability of these spleen cells to transfer an immune response from 2 to about 30% of the level obtained with NMS-treated spleen cells. Incubation of the donor spleen cells with anti-PC.1

TABLE I

Effect of Preincubation with Alloantisera and C′ on Primary Response by BALB/c Spleen Cells*

Preincubated cells‡	Serum	Added Cells	PFC/Recipient spleen§			1/Log 2 HA Titer§		
			Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Spleen	NMS	None	16,210	40,300	7,040	14.0	9.5	7.0
Spleen	Anti-θ-C3H	None	2,830	701	313	7.0	4.0	2.0
Spleen	Anti-θ-C3H	Thymus		12,125			7.5	
Spleen	Anti-PC.1	None	15,720	38,587	6,257	12.2	11.0	8.3
None		None	90	28		2.6	1.0	
None		Thymus	59			1.9		

* Preincubation for 45 min at 37°C with 1 ml of antiserum and 0.7 ml of rabbit C′ per 2×10^8 spleen cells at a final concentration of 6.7×10^6 cells per ml.

‡ 2×10^7 cells injected i.v. as determined before incubation, followed by 0.1 ml 20% SE.

§ Recipients were X-irradiated (650 R) before and sacrificed on day 6 (Exp 1) or 7 (Exps. 2 and 3) after cell transfer. HA titer = Hemagglutinin serum titer.

and C′ did not significantly affect their ability to give an immune response to SE in the recipient mice.

It was known from previous experiments (8) that, in the presence of C′, PFC of BALB/c mice are inactivated by anti-PC.1, whereas PFC of DBA/2 origin are unaffected. Additional experiments were therefore performed in order to assess the origin, donor or recipient, of the PFC in the present study. When normal DBA/2 spleen cells were used as donor cells, the recipient spleens contained somewhat less PFC on days 6–7, approximately 18–56% of the numbers seen after transfer of BALB/c cells. For the determination of the origin of the PFC, the spleen cells of recipients from both donor cell types were incubated with anti-PC.1 and C′ before analysis for the numbers of antibody-forming cells. Controls included incubation with NMS and C′. Table II presents the results obtained in two experiments. It can be seen that 75% of PFC in spleen

cell suspensions of recipients from BALB/c spleen were inactivated by exposure to anti-PC.1. No reduction in PFC in recipient spleen cells was observed after such exposure when the donors had been DBA/2, suggesting that the PFC in the recipients' spleens were indeed of donor origin.

Recipients of BALB/c spleen cells, which had been incubated with anti-PC.1 and C′ prior to transfer, also showed PFC in their spleens which were reduced by 87% after exposure to anti-PC.1 and C′. This shows clearly that, while the precursors of the PFC are resistant to this antiserum, the PFC itself is inactivated. Incubation of the PFC from recipients' spleens with anti-θ-C3H and C′ did not reduce their numbers, as had been shown previously (8).

Transfer of Secondary Response.—Immune spleen cells taken from BALB/c mice after intravenous injection of SE, and transferred in doses of 10^7 cells per recipient, gave high numbers of direct PFC in recipient spleens as early as

TABLE II

*Effect of Incubation with Anti-PC.1 on PFC in Recipient Spleens**

Recipients of	PFC/Plate after		% Reduction
	NMS + C′	Anti PC.1 + C′	
BALB/c spleen cells	327	80	75
BALB/c spleen cells preincubated with anti-PC.1	232	30	87
DBA/2 spleen cells	28	29	−3

* IgM producing PFC in spleens from immunized BALB/c mice are reduced by approximately 70% after incubation with anti-PC.1, whereas DBA/2 mice produce PFC which are resistant to this antiserum (see reference 8).

4 days after transfer (Table II), as was also observed previously (11). Incubation, with anti-θ-C3H and C′, before transfer, drastically reduced to smaller than 4% this ability to give a secondary response, and resulted in numbers of PFC per recipient spleen not different from those seen after transfer of 10^7 normal spleen cells at this time. Addition of 10^7 untreated normal spleen cells to the anti-θ-C3H–treated immune spleen cells did not reconstitute the secondary response. On the other hand, recombining anti-θ-C3H–incubated cells with anti-PC.1–treated cells resulted in a normal transfer of the secondary response, indicating that the anti-θ–treated cells did not inhibit other immune cells. Incubation with anti-PC.1 before transfer did not affect the ability of the spleen cells to give a secondary response.

Study of serum agglutinin titers on days 4–5 after transfer gave similar results. Spleen cells treated with anti-θ-C3H gave 5 to 7 log 2 lower serum titers than did immune spleen cells incubated with NMS and C′. Again no reconstitution was observed by addition of 10^7 normal BALB/c spleen cells (Table III).

In two experiments (Table IV) immune cells were taken from donor mice 2 wk after a second intravenous injection of SE and both direct and indirect PFC in recipient spleens were assayed on day 6 after transfer. Very large numbers of indirect PFC were seen at this time after transfer of cells incubated either with NMS or with the anti-PC.1 serum. Treatment with anti-θ-C3H also completely abolished this transfer of immunological memory. Attempts to reconstitute the immune capacity of the anti-θ-C3H–treated cells with 2×10^7 normal thymus or 10^7 spleen cells were relatively unsuccessful. Spleen cells did not reconstitute

TABLE III

Effect of Preincubation with Alloantisera and C′ on Secondary IgM Response by BALB/c Spleen Cells*

Preincubated‡ cells	Serum	Added cells‡	PFC/Recipient spleen§			1/Log 2 HA titer§		
			Exp. 4	Exp. 5	Exp. 6	Exp. 4	Exp. 5	Exp. 6
Immune spleen‖	NMS	None	1912	6450	4038	9.3	7.0	8.3
Immune spleen	Anti-θ-C3H	None	73	152	139	4.0	<3	1.3
Immune spleen	Anti-θ-C3H	Normal spleen		193	107		<3	2.3
Immune spleen	Anti-PC.1	None	4333	5840		10.0	6.7	
None		Normal spleen		92	138		<2	1.8

* Preincubation for 45 min at 37°C with 1 ml of antisera and 0.7 ml of rabbit C′ per 10^8 spleen cells at a final concentration of 3.4×10^6 cells per ml.

‡ 10^7 cells injected intravenously, as determined before incubation, followed by 0.1 ml of 20% SE.

§ Recipients were X-irradiated (650 R) 1 day before and sacrificed on day 5 (Exp. 4) or 4 (Exps. 5, 6) after cell transfer. Values represent average numbers of PFC and averages of 1/log 2 hemagglutinin serum titers from three to five recipients.

‖ Immune cells were obtained from mice 7 days after injection of 0.1 ml of 20% SE alone (Exp. 4) or with 10 μg E. Coli endotoxin (Exps. 5, 6).

to levels higher than those obtained with normal spleen by itself, and thymus cells reconstituted to a similar or somewhat lower level.

An additional experiment was performed to ascertain whether a large number of thymus cells would give better reconstitution. In this experiment 10^7 immune spleen cells, after incubation with NMS and C′, gave rise to 8800 direct and 50,400 indirect PFC per spleen, whereas the anti-θ-C3H–treated cells gave 2310 direct and indirect PFC per recipient spleen on day 5 after transfer. Neither addition of 2×10^7 nor of 10^8 normal thymus cells to the anti-θ–treated cells at the time of transfer had any reconstituting effect on their immunological memory for the production of direct or indirect PFC.

DISCUSSION

The results show clearly that incubation of BALB/c spleen cells with alloantiserum to θ-C3H and C′ interferes with the ability of these cells to transfer both the primary and the secondary response to SE. In the case of the primary response this effect could be partially overcome by the addition of normal thymocytes. This suggests that the anti-θ serum has specifically affected the

TABLE IV

Effect of Preincubation with Alloantisera and C′ on Secondary IgM and IgG Response by BALB/c Spleen Cells*

Preincubated cells	Serum	Added cells	PFC/Recipient spleen				1/Log 2 HA titer§	
			IgM		IgM + IgG			
			Exp. 7	Exp. 8	Exp. 7	Exp. 8	Exp. 7	Exp. 8
Immune spleen‡	NMS	None	6,000	6240	72,800	74,840	10.7	12.5
Immune spleen	Anti-θ-C3H	None	10	273	125	780	5.7	6.3
Immune spleen	Anti-θ-C3H	Normal spleen		2484		4,260		8.3
Immune spleen	Anti-θ-C3H	Normal thymus		1104		2,130		9.0
Immune spleen	Anti-PC.1	None	11,100		73,000		11.0	
None		Normal spleen		2265		3,900		6.8
None		Normal thymus		23		15		<3

* Preincubation for 45 min at 37°C with 1 ml of antisera and 0.7 ml of rabbit C′ per 10^8 spleen cells at a final concentration of 3.4×10^6 cells per ml.

‡ 10^7 cells injected intravenously, as determined prior to incubation, followed by 0.1 ml of 20% SE. Immune cells were obtained from mice 2 wk after the last of two intravenous injections of 0.1 ml of 20% SE and 10 μg E. coli endotoxin, with a 2–4 wk interval.

§ Recipients were X-irradiated (650 R) one day before and sacrificed on day 6 after cell transfer. Values represent average numbers of PFC and averages of 1/log 2 hemagglutinin serum titers from three to four recipients.

thymus-derived (TD) lymphoid cells in the spleen, as was also indicated by the observation that neonatal or adult thymectomized and anti-lymphocyte serum-treated mice have a marked decrease in the anti-θ-sensitive population (5–7). In addition, recent experiments indicate that graft-*versus*-host activity of mouse spleen cells, another function of TD cells, is abolished by incubation of cells with anti-θ serum and C′ (Takahashi, T., and E. Leckband, unpublished observations).

It is known that primary responses to certain antigens in the mouse involve both the TD and other lymphoid cells and that SE is one of these antigens. Chiller et al. (12) have shown that in the response to human γ-globulin both

cell systems are needed and, in addition, both can be rendered immune-tolerant. This implies that both lymphoid cell types have the ability to specifically recognize antigen. Experiments in the chicken also suggest that bursa cells by themselves, the prototype of thymus-independent lymphoid cells, are capable of transferring primary responses to some antigens (*Brucella abortus*) but not to others (SE) (13). Preliminary experiments with mouse spleen cells depleted of the θ-bearing population also suggest that the transferred primary response to some" thymus-independent" antigens is much less reduced than the response to SE (Mond, J., T. Takahashi, and G. J. Thorbecke, unpublished observations).

It is known that TD cells in the mouse spleen proliferate in response to SE injections (14). It is likely, although not established, that this proliferative response involves only those TD cells which are capable of interacting with SE, and results in a specific increase in the SE-reactive TD population. The present results are very much in agreement with such an increase in SE-reactive anti-θ-sensitive cells.

The question which still needs to be resolved is whether a second, non-thymus-derived cell is also needed in the secondary response. Experiments employing allotypic markers on IgG and transfer of both primary and secondary responses between congenic mouse strains have been done to study this. Although bone marrow cells enhanced the ability of spleen cells to give a transferred secondary response, the allotype of the antibody produced was always that of the spleen donor (15). Under similar experimental conditions however, Cunningham (16) found that with increasing time after the transfer there was a progressive change from spleen cell to bone marrow cell-type PFC in the recipient spleens. In addition, after transfer of a primary response with thymus and bone marrow cells, challenge to induce a secondary response in the transferred cells revealed that the IgG PFC were of bone marrow origin (15).

According to the present results the relationship between TD cells and precursors of PFC can be interpreted in several ways. (*a*) The anti-θ-sensitive cells themselves might be the memory cells becoming PFC in the secondary response, which are then no longer anti-θ-sensitive (8). (*b*) Immunization has resulted in such an increase in anti-θ-sensitive, SE-reactive cells that their effectiveness in cooperation with the non-thymus-derived cells is greatly enhanced and is, in the transfer system employed here, the major immunological memory factor.

With the cell ratios used in the present experiments, normal spleen cells did not reconstitute the ability of anti-θ-C3H-treated immune cells to transfer a secondary response of either the IgM or the IgG class. Although this finding clearly shows that the anti-θ-sensitive cells carry immunological memory, it does not exclude, by any means, the presence of immunological memory on the non-thymus-derived population. If this population is present in immune spleens in relatively great excess, then the height of the response would be determined by the thymus-derived cells under most experimental circumstances involving spleen cell transfer.

Expression of immunological memory in the precursors of the PFC could only be obtained if an artificial situation were created in which the number of these cells would be limiting for the height of the immune response. The results do not indicate that such a situation was obtained in the present experiments, even when 2×10^7 or 10^8 normal thymus cells were given together with 10^7 anti-θ–treated memory cells. The slight degree of reconstitution caused by addition of excess normal thymus on the IgM and IgG memory response in only one of two experiments was unconvincing, because the response obtained was no higher than would have been expected from a combination of anti-θ–treated normal spleen and thymus.

The class of antibody produced, which supposedly depends on the bone marrow precursor of the antibody-forming cells (17), was certainly affected by prior immunization of the spleen donors, and both the IgM and the IgG responses were equally anti-θ–sensitive. This suggests a change in the cooperating component of bone marrow origin. Experiments on sensitization to the carrier in the secondary immune response to hapten-conjugated antigens (18, 19) also suggest the presence of immunological memory both in the cells immune to the hapten and in the "helper" cells sensitive to the carrier. Again, in such systems, the class of antibody produced is determined by the cells immune to the hapten (20, 21).

No effect on the transferred primary or secondary response was obtained by incubation of the spleen cells with anti-PC.1 and C′. It should be noted that, if the precursors of PFC are normally present in excess over the TD cells, a very large percentage of these cells might have to be killed before a detectable effect on the immune response would be obtained. However, in view of the complete absence of any effect of anti-PC.1, it seems likely that the PC.1 determinant is lacking from the precursors of the PFC.

The observation that a large proportion of PFC is killed by anti-PC.1 and C′, whereas the precursors of the antibody-forming cells appear resistant in the present system, suggests that the PC.1 antigen is a determinant acquired during differentiation into PFC. The fact that not all PFC in BALB/c mice are inactivated by the anti-PC.1 and that indirect PFC are more sensitive than direct PFC further suggests that this surface antigen is acquired relatively late during the differentiation into the antibody-secreting cell.

It may be of interest, in this respect, to mention that experiments on the secondary immune response to SE by chicken spleen cells in vitro have given similar results to the ones presented here. Rabbit antisera specifically directed against thymus strongly interfere with the secondary response in vitro, whereas antisera specifically directed against bursa are much less effective in this respect (22).

Recent publications strongly suggest that those lymphoid cells which are induced to proliferate upon exposure to anti-immunoglobulin antisera are non-thymus-derived in the rabbit (23) and in the chicken (24). In view of the above

results it appears quite important to find a means for the elimination of the non-thymus–derived precursors of PFC in the mouse. The percentages of lymphoid cells in a variety of lymphoid organs of mice killed by anti-θ and by anti-k antisera complement each other and, when combined, represent approximately 100% of the cells (Takahashi, T., L. J. Old, R. K. McIntire, and E. A. Boyse, unpublished observations). Therefore, attempts are now underway to affect the adoptive immune responses with anti-immunoglobulin antisera.

SUMMARY

Spleen cell transfer studies were done in BALB/c strain mice in an attempt to define the role of θ-antigen–bearing lymphoid cells in immune responses to SE. Incubation with alloantiserum to θ-C3H and rabbit C′ virtually completely abolished the ability of the cells to transfer both primary and secondary (IgM and IgG) responses to 650 R irradiated recipients. Normal thymus cells partially reconstituted the ability of such treated cells to transfer the primary but not the secondary response. The results are interpreted as showing immunological memory for SE in the θ-bearing thymus-derived cells.

Incubation of the spleen cells with alloantiserum to the PC.1 antigen present on antibody-forming cells did not significantly affect the ability to transfer either primary or secondary response.

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