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THE HUMORAL IMMUNE RESPONSE TO ALLOGRAFTS OF FOETAL SMALL INTESTINE IN MICE

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Summary.—The influence of the presence of " passenger leucocytes " on the production of anti H-2 antibodies has been studied in mice receiving allografts of foetal small intestine, adult skin or intradermally injected spleen cells. It was found that the humoral immune response to foetal intestine (a tissue without passenger leucocytes) was identical temporally to that elicited by skin allografts and these responses differed from that following injection of allogeneic spleen cells in that antibodies to solid grafts took longer to appear. The humoral immune response to small intestine grafts was not evident until several days after the onset of graft rejection as assessed morphologically. Anti H-2 antibody production was not observed in thymus deprived recipients of foetal small intestine allografts or allogeneic spleen cells, and this suggests that the humoral immune response to transplantation antigens is thymus dependent.

AN ALLOGRAFT of tissue between individuals disparate for antigens of the major histocompatibility (H) system leads to sensitization of the cell mediated immune response and also to the production of anti-H antibodies. The form of the sensitizing antigen is still a matter of controversy and it is not clear whether the immunizing antigen is in the same form for both types of immune response. " Passenger leucocytes " in tissue grafts have been suggested as a major source of immunizing stimulus in skin (Steinmuller, 1967, 1969; Steinmuller and Hart, 1971), and renal allografts (Guttman, Linquist and Ockner, 1969; Stuart *et al.*, 1971). It has also been suggested that leucocytes are the immunizing antigen in local graft-versus-host reactions (Elkins, 1966). On the other hand, there is evidence that the humoral immune response is stimulated by antigenic debris released from a graft undergoing rejection (Monaco and Franco, 1969; Elves, 1971).

The foetal mouse intestine graft offers a system with which this problem may be explored as it contains virtually no leucocytes when implanted (Ferguson and Parrott, 1972*a*, *b*). The morphological appearances of partial and complete rejection have been defined previously (Ferguson and Parrott, 1973).

We have studied the humoral immune response after implantation of foetal small intestinal allografts in H-2 disparate hosts, and report here that a humoral response occurs in these mice in the absence of passenger leucocytes. We have also examined the time course of appearance of anti-H antibody in order to find out if the appearance of antibody precedes, coincides with or follows the morphological changes of partial and complete rejection, and we have studied the thymus dependence of this antibody response in a small group of thymus deprived mice.

MATERIALS AND METHODS

Animals and operative techniques.—Mice of the inbred CBA (H-2^k) and BALB/c (H-2^d) strains were used. These animals were maintained in the Department of Bacteriology and Immunology, University of Glasgow.

Grafts of small intestine.—The techniques for implantation of grafts of foetal small intestine have been described in detail elsewhere (Ferguson and Parrott, 1972*a*; Ferguson, 1973). Fragments of small intestine from 18-day mouse foetuses were implanted under the kidney capsules of adult recipient mice (2 grafts per recipient). At this stage of gestation there are neither lymphocytes nor plasma cells in the lamina propria of mouse intestine (Ferguson and Parrott, 1972*a*, *b*). Rarely, rudimentary Peyer's patches are present and these comprise mainly reticulum cells.

Grafts of skin.—Two pieces of tail skin were grafted in beds prepared in the dorsal skin of recipient mice; they were protected by encasing the recipient's trunk in a cuff of plaster of Paris.

Transfer of spleen cells.—Suspensions of spleen cells were prepared by gently squeezing spleen fragments through a sieve, further dissociating the suspension in Eagle's medium. 1×10^7 cells (in volume of 0·05 ml) were then injected intradermally into the dorsal skin of recipient mice.

Preparation of thymus deprived mice.—Thymus deprived mice were thymectomized at 5 weeks of age and 2 weeks later were given 850 rad whole body x-irradiation, with lead shielding of the feet to allow autorepopulation by marrow cells (Parrott and Ferguson, 1974).

Animals were killed at intervals after grafting, or immunization, and blood was collected from the antecubital fossa. Serum was separated and stored frozen at −20° until tested.

Assessment of rejection in grafts of small intestine.—Grafts were dissected, cut, fixed in formol saline and paraffin embedded. 4 μm thick sections were cut at 3 levels of the tissue and stained with haemotoxylin and eosin and with methyl green pyronin, to allow morphological grading of the stage of rejection. Sections were coded and assessed, " blind " into one of 3 categories:

1. Normal (*i.e.* the usual morphology of neonatal mouse small intestine; virtually no lymphoid cells outside the Peyer's patches); 2. Established rejection (infiltration of the tissue with lymphoid cells; epithelial integrity is maintained, although in many specimens there is villous atrophy with crypt hyperplasia (grades L+, L++ and FLAT in previous paper)); 3. Complete rejection (the mucosa is destroyed, with no lining epithelium; the tissue consists of smooth muscle and debris, heavily infiltrated with lymphoid cells).

Examination of serum for antibody.—Sera were thawed and decomplemented before being dispensed in 2 μl aliquots on to microplates under paraffin oil. A fluorochromatic cytotoxic test was then employed to examine each serum for antibodies (Elves, 1973); lymphocytes from pooled lymph nodes (axillary, brachial, cervical and mesenteric nodes) were used as target cells. The nodes were teased apart, the cells released by repeated aspiration into a 5 ml syringe and then washed 3 times in TC199, before being made fluorescent with fluorescein diacetate. Rabbit serum was used as a source of complement.

RESULTS

Time course of rejection of allografts

The results of histological examination of allografts of small intestine in animals

TABLE I.—*Histological Grading of Rejection of Small Intestinal Allografts*

Donor	Host	Days after grafting	No. of grafts*			No. of mice	
			Examined	With established rejection	With complete rejection	Examined	With complete rejected graft
CBA	BALB/c	3	4	4	—	2	0
		5	12	12	—	6	0
		7	11	6	5	6	4
		9	12	1	11	6	6
		12	12	1	11	6	6
		15	11	—	11	6	6
BALB/c	CBA	3	4	3	—	2	0
		5	10	10	—	6	0
		7	12	10	2	6	2
		9	10	4	6	6	5
		12	12	1	11	6	6
		15	12	1	11	6	6
BALB/c	TxCBA	9	4	1	—	2	0
		15	10	5	—	5	0
		25	4	1	2	2	1

* Each mouse received 2 grafts. Tx = Thymectomized irradiated.

TABLE II.—*Incidence of Cytotoxic Antibody Production in Mice Receiving Allografts of Small Intestine, Skin or Allogeneic Spleen Cells*

Type of graft	Host	Donor	No. of positive/total number tested on days						
			3	5	7	9	12	15	25
Small intestine	CBA	BALB/c	0/2	0/6	0/6	0/6	6/6	6/6	—
Small intestine	BALB/c	CBA	0/2	0/6	0/6	0/6	6/6	6/6	—
Small intestine	TxCBA	BALB/c	—	—	—	0/2	—	0/5	0/2
Skin	CBA	BALB/c	—	0/1	0/1	0/7	3/3	—	—
Spleen cells	CBA	BALB/c	—	—	5/6	—	6/6	—	—
Spleen cells	TxCBA	BALB/c	—	—	0/3	—	0/6	—	0/6

Tx = Thymectomized irradiated.

killed at various intervals are shown in Table I.

Evidence of rejection was seen in some grafts as early as 3 days after grafting and by the fifth day all grafts showed established rejection. By the ninth day, in the case of BALB/c recipients, or the twelfth day, in the case of CBA recipients, rejection was complete in all but 2 grafts. Grafts in thymus deprived mice showed less evidence of rejection and even after 25 days one of 4 grafts still showed no evidence of rejection; both completely rejected grafts were in the same mouse.

Morphological examination of skin grafts was not carried out. However, in other experiments carried out between these CBA and BALB/c strains, skin graft rejection occurred predictably at 10 or 11 days.

Humoral immune response to allografts and to allogeneic cells

The incidence of cytotoxic antibodies in recipients of small intestine or skin grafts is shown in Table II. For comparison, data from recipients of allogeneic spleen cells are also shown.

No antibodies were found in the small intestine allograft recipients of either strain which were killed up to and including the ninth day. By Day 12, however, all recipients possessed antibody. The pattern of antibody production in recipients of skin allografts was similar, with no antibody being detectable before the twelfth day. In contrast, 5 out of 6 animals immunized with spleen cells had developed serum antibodies by the seventh day after injection.

Thymus deprived mice failed to show any antibody production at any time up to 25 days, against either small intestine allografts or allogeneic spleen cells.

DISCUSSION

The results presented above show that cytotoxic anti-H antibodies do not appear in the serum of recipients of foetal intestine grafts until some days after the onset of rejection and, in the case of all but 2 of the animals studied, not until the grafts were completely rejected. The same temporal relationship between graft rejection and appearance of antigraft antibody was found in skin graft recipients and this is similar to our previous experience with skin grafts in rats (Elves, 1971, 1974), that of Rolstad, Williams and Ford (1974) and that of Monaco and Franco in mice (1969). However, the experiments in which animals were immunized with spleen cells showed that the tempo of antibody production was accelerated when antigens gained immediate access to the lymphoid tissues, and once again this is similar to findings in the rat (Elves, 1974; Rolstad *et al.*, 1974). We have already produced evidence that the delayed appearance of antibody to skin grafts cannot be attributed to adsorption of antibody by the grafts, for the time course of antibody production is unaltered by premature removal of the graft (Elves, 1971). Now we report that antibody production follows the expected course when there

are no passenger leucocytes in the allograft, and these results support the theory that the humoral immune response is stimulated by debris released from partially rejected solid tissue allografts (Monaco and Franco, 1969; Elves, 1971).

Thus, it may be suggested that in the case of tissue allografts the antigenic stimulus to antibody production is distinct and delayed from that which sensitizes the cell mediated immune response. The identical response in recipients of skin grafts and recipients of leucocyte depleted intestine grafts suggests that " passenger leucocytes " are not the stimulus for antibody production. Furthermore, the fact that cell mediated immunity is also aroused in intestine graft recipients leads to the conclusion that passenger leucocytes are not a principal antigenic stimulus in this context either. These results do not support Steinmuller's hypothesis that passenger leucocytes are a major source of immunizing antigen (Steinmuller, 1969; Steinmuller and Hart, 1971). This was based upon experiments using isografts from chimaeric mice. However, there is some evidence that the fixed elements of the dermis may be derived from blood-borne precursor cells and are therefore also chimaeric (Fichtelius and Diderholm, 1962; Gillman and Wright, 1966; Barnes and Khruschov, 1968). As Steinmuller himself has pointed out, this could be an alternative explanation of his findings and would not require passenger leucocytes.

T cell depleted mice did not produce any antibody after injection of allogeneic spleen cells and this finding clearly indicates that the humoral response to H-2 antigens is thymus dependent. Thymus depleted recipients of intestine grafts also failed to develop antibody despite the fact that 4 of 14 grafts removed after 15 or 25 days showed morphological evidence of a cell mediated response. Thus, this is further evidence that antibody production in response to transplantation antigens is thymus dependent. Rolstad and his colleagues have similarly shown the thymus dependence of the humoral response to H-antigens in the rat (Rolstad *et al.*, 1974). However, these results are at variance with those obtained by Monaco and Franco (1969), who found that adult thymectomized mice treated with anti-lymphocyte serum produced anti-H antibody after the eventual rejection of a skin graft. However, in these experiments if the graft was not rejected, then no antibody was formed. It is quite possible that the differences between these two groups of experiments are due to the time at which animals were studied; we have examined T deprived mice up to 25 days after grafting.

The morphology of partial rejection of small intestinal allografts resembles that of coeliac disease in man (Holmes *et al.*, 1971; Ferguson and Parrott, 1973). Villi are short or absent, crypts of Lieberkuhn are abnormally long and there is infiltration of the lamina propria with many lymphoid cells. Although rejection is thymus dependent (Ferguson and Parrott, 1973), it has not previously been established whether this " subtotal villous atrophy " of rejection is produced by the cellular or the humoral immune response. This point has been resolved by the present series of experiments because the typical morphology of subtotal villous atrophy was present in 16 of the grafts examined between 3 and 9 days after implantation, although none of the host mice had cytotoxic antibodies in the serum. These striking effects of rejection on crypts and villi can therefore be attributed to the cellular component of the immune response to the allograft. This finding adds support to the theory that local cell mediated immune response may cause crypt hyperplasia and villous atrophy in human and animal disease—*e.g.* coeliac disease (Ferguson, 1974) and helminth infection of the small intestine (Ferguson and Jarrett, 1975).

In conclusion, this investigation has shown that foetal small intestine allografts resemble skin allografts in that the humoral immune response is temporally dissociated from the cell mediated re-

sponse, and suggests that the antigens which stimulate antibody production are liberated from the damaged graft. Moreover, it is clear that in these mice the humoral immune response to transplantation antigen is thymus dependent.

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REFERENCES

BARNES, D. W. H. & KHRUSHCHOV, N. G. (1968) Fibroblasts in Sterile Inflammation: Study in Mouse Radiation Chimaeras. *Nature, Lond.*, **218**, 599.

ELKINS, W. L. (1966) The Interaction of Donor and Host Lymphoid Cells in the Pathogenesis of Renal Cortical Destruction induced by a Local Graft versus Host Reaction. *J. exp. Med.*, **123**, 103.

ELVES, M. W. (1971) The Afferent Arc: The Route of Sensitisation in the Homograft Reaction. In *Immunological Tolerance to Tissue Antigens.* Oswestry: Orthopaedic Hospital. p. 1.

ELVES, M. W. (1973) An Evaluation of a Modified Fluorochromatic Technique for the Detection of Cytotoxic Antibodies. *J. immun. Methods*, **2**, 129.

ELVES, M. W. (1974) Humoral Immune Response to Allografts of Bone. *Int. Arch. Allerg. Appl. Immunol.*, **47**, 709.

FERGUSON, A. (1973) Implantation of Tissue under the Kidney Capsule. In *Handbook of Experimental Immunology.* Oxford: Blackwell. p. A312.

FERGUSON, A. (1974) Lymphocytes in Coeliac Disease. In *Coeliac Disease.* Leiden: Stenfert Kroese. p. 265.

FERGUSON, A. & JARRETT, E. E. E. (1975) Hyposensitivity Reaction in the Small Intestine. I. Thymus-Dependence of Experimental "Partial Villous Atrophy". *Gut*, **16**, 114.

FERGUSON, A. & PARROTT, D. M. V. (1972*a*) Growth and Development of "antigen-free" Grafts of Foetal Mouse Intestine. *J. Path.*, **106**, 95.

FERGUSON, A. & PARROTT, D. M. V. (1972*b*) The Effect of Antigen Deprivation on Thymus-dependent and Thymus-independent Lymphocytes in the Small Intestine of the Mouse. *Clin. & exp. Immunol.*, **12**, 477.

FERGUSON, A. & PARROTT, D. M. V. (1973) Histopathology and Time Course of Rejection of Allografts of Mouse Small Intestine. *Transplantation*, **15**, 546.

FICHTELIUS, K. K. & DIDERHOLM, H. (1961) Autoradiographic Analysis of the Accumulation of Lymphocytes in Wounds. *Acta. path. microbiol., scand.*, **52**, 11.

GILLMAN, T. & WRIGHT, L. J. (1966) Autoradiographic Evidence suggesting *in vivo* Transformation of some Blood Mononuclears in Repair and Fibrosis. *Nature, Lond.*, **209**, 1086.

GUTTMAN, R. D., LINQUIST, R. R. & OCKNER, S. A. (1969) Renal Transplantation in the Inbred Rat. *Transplantation*, **8**, 472.

HOLMES, J. T., KLEIN, M. S., WINAWER, S. J. & FORTNER, J. G. (1971) Morphological Studies of Rejection in Canine Jejunal Allografts. *Gastroenterology*, **61**, 693.

MONACO, A. P. & FRANCO, D. J. (1969) The Effect of Heterologous Antilymphocyte Serum on the Formation of H-2 Antibody and the Induction of Allograft Immunity. *Transplantn Proc.*, **1**, 474.

PARROTT, D. M. V. & FERGUSON, A. (1974) Selective Migration of Lymphocytes within the Mouse Small Intestine. *Immunology*, **26**, 571.

ROLSTAD, B., WILLIAMS, A. F. & FORD, W. L. (1974) The Alloantibody Response to a strong Transplantation Antigen (Ag-B). Quantitative Aspects and Thymus-dependence of the Response. *Transplantation*, **17**, 416.

STEINMULLER, D. (1967) Immunization with Skin Isografts taken from Tolerant Mice. *Science, N.Y.*, **158**, 127.

STEINMULLER, D. (1969) Allograft Immunity Produced with Skin Isografts from Immunologically Tolerant Mice. *Transplantn Proc.*, **1**, 593.

STEINMULLER, D. & HART, E. A. (1971) Passenger Leucocytes and Induction of Allograft Immunity. *Transplantn Proc.*, **3**, 673.

STUART, F. P., BASTIEN, E., HOLTER, A., FITCH, F. W. & ELKINS, W. L. (1971) Role of Passenger Leucocytes in the rejection of Renal Allografts. *Transplantn Proc.*, **3**, 461.