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Acquisition of immunological self-recognition by the fetal rat

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SUMMARY

Rats in which normal development of the thyroid gland had been interrupted by the injection of ^{131}I during fetal life are liable to mount autoimmune responses against grafts of syngeneic thyroid tissue transplanted in adult life. Although autoimmune thyroiditis developed spontaneously in grafted tissue, the recipients' own thyroid glands remained free from autoimmune changes, showing only irradiation damage. Other syngeneic endocrine grafts transplanted to these rats were not susceptible to autoimmune attack. This experiment demonstrates that contact of self-determinants with the developing mammalian immune system is required if autoimmunity is to be prevented.

INTRODUCTION

The ability to discriminate between self and non-self is fundamental in regulating autoimmunity. Whilst there has been much speculation concerning the subject of self-recognition by the immature immune system, there has been little experimentation. Two experimental attempts to interfere with the acquisition of self-tolerance by amphibians have been reported (Triplett, 1962; Rollins-Smith & Cohen, 1982). Each experiment entailed removal of an organ at the larval stage, its temporary transplantation to another host of the same species and its relocation in the donor at a later stage of maturation. The experimental hypothesis was that, if absence of anti-self reactivity in mature animals was predicated upon self-exposure of the immature immune system, then removal of any self-antigen sufficiently early in development would prevent development of tolerance of it on the part of the bearer. The original experiments indicated that a majority of pituitary isografts that had been 'stored' in intermediate hosts were rejected when replaced in the donor tree frog (*Hyla regilla*) 2 months later (Triplett, 1962). However, when the experiment was repeated using *Rana pipiens*, uniform acceptance of reimplanted grafts occurred (Rollins-Smith & Cohen, 1982).

Two limitations in these experiments with larval amphibians complicate their interpretation. Alloreactivity of the donor/definitive host against intermediate host-derived tissue inadvertently re-implanted with the pituitary graft may have produced false positive graft rejection in Triplett's (1962) experiments (cited by Rollins-Smith & Cohen, 1982). Re-implanted pituitary grafts of Rollins-Smith & Cohen (1982), which contained considerably less intermediate host tissue, were never rejected. On the other hand, graft rejection may have failed to occur in either experiment because of immaturity of the recipient's immune system at the time of pituitary re-implantation. As frogs in both experiments remained sufficiently immature at the time of reimplantation to accept some *allogeneic* pituitary grafts, the risk of false negative results attributable to *de novo* induction of tolerance by reimplanted self-grafts could not be excluded.

This paper reports a novel approach to the investigation of self-tolerance in mammals. This entails the infliction of damage on the fetal rat thyroid gland using radioactive iodine, followed, in adult life, by the implantation into treated rats of thyroid tissue from syngeneic donors. Both of the limitations of the earlier reports are circumvented. Use of syngeneic graft tissue excludes any possibility of graft damage as a result of alloreactivity against contaminating tissue from an intermediate host. Deferral of graft re-implantation until the recipient had attained adult life eliminates any risk that the graft itself could prove to be tolerogenic.

The results indicate that syngeneic thyroid grafts become liable to autoimmune attack in rats in which thyroid development has been subject to earlier interference. This finding accords with that of a related experiment in fetal lambs, involving removal of the thyroid gland at a stage when induction of allograft tolerance remains possible. Subsequent replacement of the thyroid gland in its donor, when the immune system had matured, evoked a vigorous immunological assault (P. McCullagh, unpublished results).

MATERIALS AND METHODS

Animals

Rats of the inbred DA strain (Animal Breeding Establishment, John Curtain School of Medical Research) were used throughout these experiments. Matings were timed by observation of vaginal plugs, the morning on which these were detected being designated as Day 0.

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Administration of ^{131}I

^{131}I was administered to fetal rats using a tuberculin syringe mounted in a remote control stereotaxic apparatus. Each 2-mCi batch of ^{131}I (New England Nuclear, Boston, MA) was diluted to 1 ml in sterile distilled water and a dose of 50 μl (100 μCi) injected into the peritoneal cavity of each fetus under direct vision.

Inplantation

Syngeneic thyroid, adrenal and pituitary grafts were implanted under the renal capsule under ether anaesthesia. A small incision was made in the renal capsule and, while the edge of this incision was held with forceps, the graft was introduced and moved away from the incision. Care was taken to ensure that the graft was not lost from the incision during replacement of the kidney in the abdomen.

Radioimmunoassay

Thyroid-stimulating hormone (TSH), thyroglobulin and anti-thyroglobulin antibody in serum were measured by radioimmunoassay. TSH was assayed using a National Institute of Arthritis and Metabolic Disease (Bethesda, MD) rat TSH kit. Thyroglobulin was extracted from rat thyroid glands and purified as described by Weetman *et al.* (1982). It was labelled with ^{125}I using lactoperoxidase, as described by Zanetti & Bigazzi (1981). After filtering through a G-175 column, the contents of the peak elution tube were used for radioimmunoassay by the method of Izumi & Larsen (1978). Anti-rat thyroglobulin antibody was measured using Zanetti & Bigazzi's (1981) modification of the technique of Salabe, Fontana & Andreoli (1972).

Experimental plan

Fetal DA rats of from 15 to 18 days gestation were exposed by laparotomy of the ether-anaesthetized mother. Each fetus was inoculated i.p. with 100 μCi of ^{131}I. While a majority of fetuses survived this procedure, their post-natal physical development was usually delayed. Apart from retardation of development, no features distinctive of hypothyroidism were noted. Lymphoid tissues appeared morphologically normal. Non-specific immune responsiveness was not tested. Thyroxine was not administered to any of these rats. No further procedures were undertaken on these rats until adult life, when serum was collected for measurement of thyroglobulin and anti-thyroglobulin antibody. At this time, one or two lobes of thyroid gland from a syngeneic donor of the same sex were implanted under the renal capsule of each rat. Three to six weeks later, all recipients were bled out and both thyroid grafts and host thyroid glands were removed for histological examination. Tissues were fixed in 10% neutral formalin, dehydrated through alcohol and embedded in paraffin. Four-micrometres thick sections taken at 80-μm intervals were stained with haematoxylin-eosin (H&E) and examined by light microscopy.

Inflammatory changes in thyroid tissue were classified into five grades, as in experimental allergic thyroiditis (EAT), namely: (1) occasional foci of lymphocytic infiltration; (2) multiple foci with follicular destruction; (3) coalescing foci; (4) multiple foci accompanied by extensive areas of continuous lymphocytic infiltration; (5) total infiltration of thyroid tissue.

Table 1. Influence of gender and age of exposure to ^{131}I on severity of thyroiditis developing in syngeneic thyroid grafts

Gestational age of ^{131}I exposure (days)	Gender	No. of rats	Grade of thyroiditis in graft (mean ± SE)
15–16	M	18	1·06 ± 0·13*
	F	16	0·87 ± 0·16
17–18	M	14	2·57 ± 0·44*†
	F	11	0·73 ± 0·19†

The severity of thyroiditis was graded on the basis of examination of histological sections as indicated in the Materials and Methods. All thyroid grafts, including those which remained normal in appearance, were used in calculating mean severity of thyroiditis in a group of rats. For this purpose, unaffected grafts were scored as grade 0.

Significance of differences $P < 0·01$ between pairs * and †.

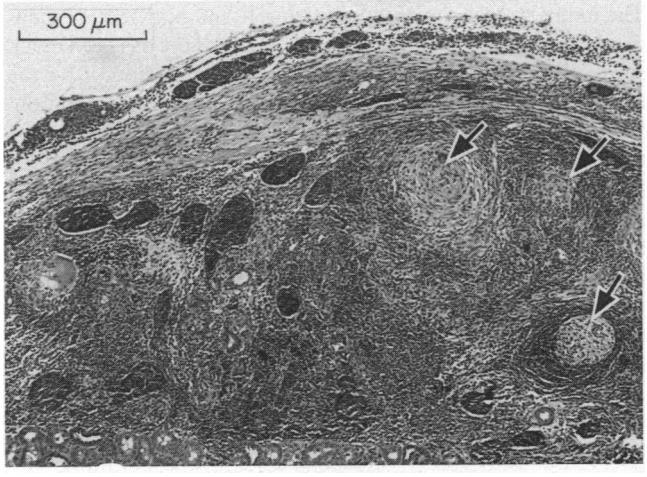

Figure 1. Thyroid graft placed under renal capsule of ^{131}I-treated syngeneic recipient. Note heavy lymphocytic infiltration and germinal centre formation (arrowed). H & E.

RESULTS

A total of 59 rats, exposed to ^{131}I *in utero*, received syngeneic thyroid implants as adults. Inflammation (from grades 1 to 5) with lymphocytic infiltration and follicular destruction was observed in the thyroid grafts of 40 of these rats. Table 1 summarizes the results of their histological examination.

Complete destruction of thyroid follicles with formation of adventitious lymphoid follicles and germinal centres occurred in the more severe reactions (Fig. 1). Multinucleate giant cells were prominent in some grafts (Fig. 2). In contrast, syngeneic thyroid grafts implanted in two control groups of previously untreated rats—seven female and 10 male—remained completely free from inflammation. When examined 3–6 weeks later, there was

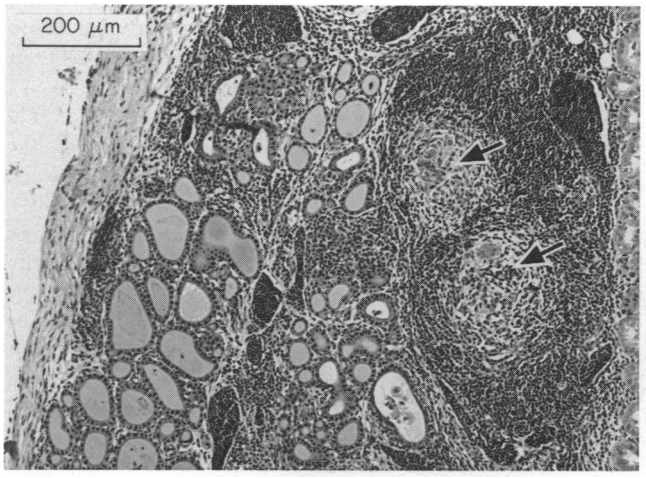

Figure 2. Graft as in Fig. 1. Note giant cell formation (arrowed). H & E.

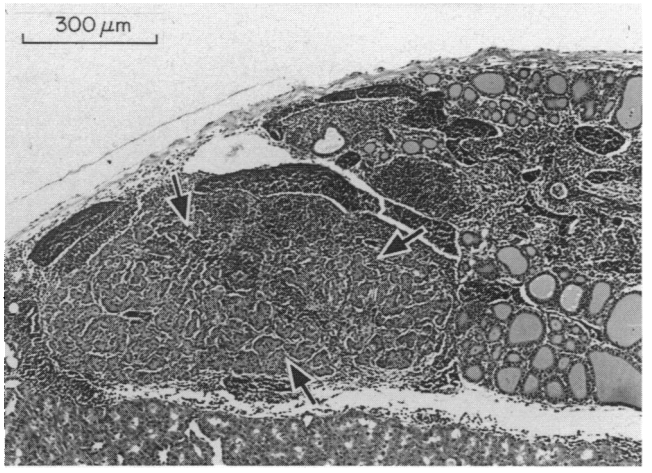

Figure 4. Parathyroid gland transferred, together with thyroid graft, to ^{131}I-treated syngeneic recipient. Note heavy lymphocytic infiltration of surrounding thyroid tissue with preservation of normal parathyroid (arrowed, same thyroid graft as illustrated in Figs 1 and 2). H & E.

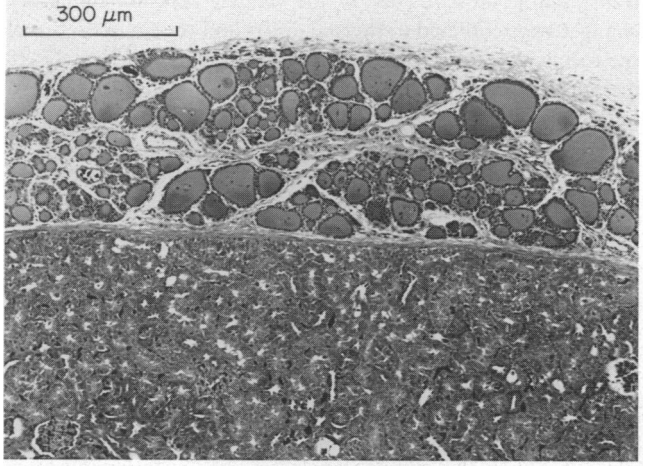

Figure 3. Thyroid graft under renal capsule of otherwise untreated syngeneic recipient. H & E.

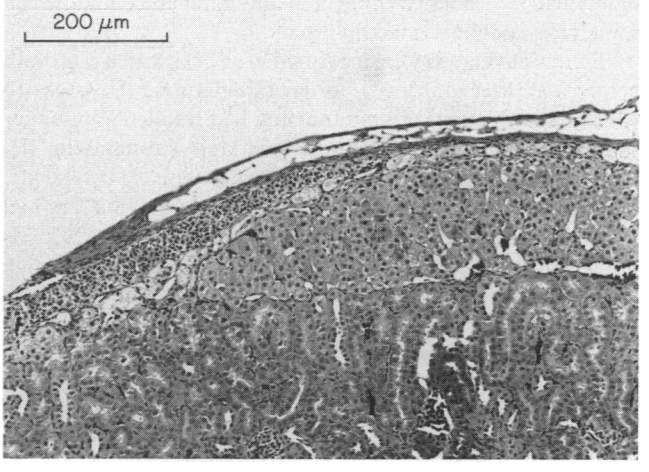

Figure 5. Adrenal graft placed under renal capsule of ^{131}I-treated syngeneic recipient. Note normal appearance of zona fasciculata of adrenal overlying renal cortex. H & E.

no indication of lymphocytic infiltration or inflammation in any of these grafts (Fig. 3).

Inflammatory changes were significantly more severe in grafts placed in male recipients. The mean grade of thyroiditis was $1{\cdot}78 \pm 0{\cdot}24$ in 32 male rats exposed to ^{131}I over the total period, 15–18 days, compared with $0{\cdot}80 \pm 0{\cdot}12$ in grafts in 27 similarly prepared females ($P < 0{\cdot}01$). (Rats with histologically normal grafts were included and scored as grade 0 in calculating these means). As indicated in Table 1, thyroiditis was much more severe in males inoculated with ^{131}I at 17–18 rather than at 15–16 days gestation. Every graft placed in the 14 males that had been exposed to ^{131}I at 17–18 days developed thyroiditis equal to or greater than grade 2.

Inflammatory changes in renal subcapsular grafts were specific for thyroid tissue. Concomitantly grafted parathyroid tissue was spared (Fig. 4). Of the thyroid grafts placed in ^{131}I-treated rats, 20 included a substantial portion of parathyroid gland. However, only one parathyroid graft was affected by the accompanying thyroiditis. In this exceptional case, heavy infiltration of surrounding thyroid and adipose tissue extended in the form of lymphocytic penetration of parathyroid interlobular connective tissue. However, parathyroid parenchyma remained normal.

As a further control on the organ specificity of the inflammatory changes, seven ^{131}I-treated rats received renal subcapsular grafts of syngeneic pituitary and adrenal glands, as controls. Whilst inflammation in the accompanying thyroid grafts in these seven recipients ranged from grade 1 to grade 4, lymphocytic infiltration remained totally absent from all other grafted tissues. (Fig. 5).

The host thyroid gland of every ^{131}I-treated rat was examined for evidence of autoimmune inflammation. Irrespective of the severity of lymphocytic infiltration of syngeneic thyroid grafts placed in the same rat, host thyroid glands were invariably

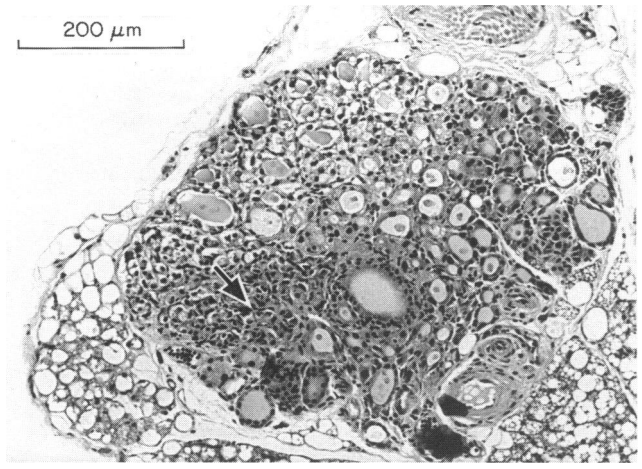

Figure 6. Host thyroid gland in ^{131}I-treated rat. Note marked atrophy of thyroid tissue with absence of lymphocytic infiltration. Intact parathyroid gland in left lower quarter (arrowed). H & E.

unaffected by this process. Host thyroids showed varying degrees of radiation necrosis, fibrosis and atrophy (Fig. 6). Thyroid follicles persisting in radiation-damaged glands commonly had low inactive epithelium and minimal colloid, which sometimes contained macrophages.

Serum TSH levels were measured in all ^{131}I-treated rats, both before and after grafting, in order to determine the extent to which impairment of thyroid function had resulted in pituitary stimulation (Table 2). TSH was invariably substantially elevated before thyroid grafting in rats treated with ^{131}I in fetal life. For example, the mean TSH level in the 14 male rats that had received ^{131}I at 17–18 days was approximately 10 times the levels observed in normal rats (these were not significantly different in male and female rats). Thyroid grafting reduced TSH levels in these ^{131}I-treated rats to the normal range.

Serum thyroglobulin levels of these rats before grafting were within the normal range (Table 2). Thyroglobulin levels in the serum of rats that had been exposed to ^{131}I at 17–18 days increased significantly ($P<0.01$) after grafting. Serum thyroglobulin was elevated above the normal range in rats exposed at 15–16 days before grafting, and was not significantly altered by this procedure. This release of thyroglobulin into the circulation after grafting probably reflects the autoimmune-mediated graft damage that was evident histologically in these rats.

Thyroxine was present in the serum of rats exposed to ^{131}I in fetal life, albeit at substantially reduced levels. Whereas the mean titre in 10 normal DA rats was 73.0 ± 5.0 ng/ml, 10 treated rats showed a mean titre of 39.1 ± 3.0 ng/ml ($P<0.001$). Following transplantation of syngeneic thyroid tissue, this rose significantly to 52.3 ± 3.2 ng/ml ($P<0.01$). Anti-thyroglobulin antibody titres were measured in every rat in order to determine whether autoimmune reactivity against thyroglobulin could be responsible for the damage sustained by the grafts. However, antibody remained undetectable in all rats, both before and after grafting, implying that thyroglobulin was not the target of the autoimmune reaction against the thyroid grafts.

DISCUSSION

The administration of ^{131}I to fetal DA rats resulted in the development of lymphocytic infiltration and varying degrees of follicle destruction in syngeneic thyroid grafts when these were transplanted subsequently to the treated rats. Inflammatory changes were confined to thyroid grafts and were more severe if the recipient was male. Exposure of recipients to ^{131}I at 17–18 days gestation was associated with significantly higher grades of inflammation in grafted thyroid tissue than exposure at 15–16 days.

The morphological similarity to EAT of the inflammatory changes in thyroid grafts in ^{131}I-treated rats suggests an autoimmune basis as the most likely explanation. The restriction of inflammation to thyroid grafts in these rats, with sparing of grafted parathyroid, pituitary and adrenal tissue, indicates that sensitization was organ specific. Furthermore, the completely normal morphology of thyroid tissue transplanted to normal rats confirms that interference with thyroid development by exposure to ^{131}I in fetal life is essential for sensitization.

As regards the sequence of sensitization after ^{131}I exposure *in utero*, it is likely that this process is triggered by implantation of the thyroid graft. The alternative possibility that sensitization results from release of antigenic material from radiation-damaged host thyroid gland much earlier in life is not supported

Table 2. Serum TSH and thyroglobulin levels before and after thyroid grafting, in rats which had been exposed to ^{131}I *in utero*

Gestational age of ^{131}I exposure (days)	Gender	No. of rats*	Serum TSH (ng/ml)		Serum thyroblogulin (ng/ml)	
			Pre-graft	Post-graft	Pre-graft	Post-graft
15–16	M	18	24·7 ± 3·5†	4·8 ± 0·6	664 ± 215	757 ± 67
	F	16	14·7 ± 2·4†	4·8 ± 0·3	1243 ± 397	624 ± 81
17–18	M	14	33·4 ± 5·9‡	5·0 ± 0·3	255 ± 33§	988 ± 191§
	F	11	17·2 ± 4·1	4·6 ± 0·4	252 ± 24¶	744 ± 138¶

* Groups of rats are identical with those reported in Table 1. Normal range of serum levels; TSH 2·5–5·0 ng/ml; thyroglobulin: 180–400 ng/ml.

Significance of differences: $P<0.01$—§, ¶; $P<0.05$—†, ‡.

by the total absence of autoimmune inflammation in the host tissue. Consequently, secondary autoimmune damage to grafted thyroid tissue, in the manner that is believed to occur after tissue damage in symphathetic ophthalmia (Gass, 1982), is an unlikely explanation.

The present experiments have not identified the antigen that is the target of the autoimmune process. A number of possible candidate antigens exist in the thyroid gland (Doniach, 1975). However, the consistent absence of indications of autoimmunity directed against persisting host thyroid tissue suggests that ^{131}I-mediated interference with development eliminates the target determinant(s) of the autoimmune process. The complete absence of anti-thyroglobulin antibody from the serum of ^{131}I-treated rats at any time, in contrast with its regular presence in rats developing EAT after immunization with thyroglobulin, implies that this is not the target antigen in the present experiments. A further indication that thyroglobulin is not the target antigen was provided by the presence of normal serum thyroglobulin levels in ^{131}I-treated rats before grafting. The presence of thyroxine, albeit at reduced levels, in the serum, likewise indicates the persistence of thyroid function, despite radiation-induced damage. No explanation for the high levels of thyroglobulin detected before grafting in rats exposed at 15–16 days can be given. It might be expected that a self-antigen against which autoimmunity had developed because of its inadequate production during development would remain undetectable. This may not be an absolute restriction, given that immune responses against growth hormone have been observed to follow its therapeutic administration to pituitary dwarfs, even though this hormone was detectable in the serum before therapy (Illig, 1970).

The occurrence of the most severe thyroiditis in grafts in recipients treated with ^{131}I at 17–18 days was as predicted, as the fetal rat thyroid first acquires the ability to concentrate iodine at 17 days and 11 hr of pregnancy (Remy *et al.*, 1980). It is probable that a high proportion of any ^{131}I administered before this time would be sequestrated in the maternal thyroid. Damage was produced in the thyroid glands of rats exposed at the earlier age, and the elevated serum TSH levels in these animals indicates substantial impairment of thyroid function. Neverthelesss, the lower levels of thyroiditis that developed in grafts placed in these rats may have reflected a lower level of interference with expression of the target antigen(s) during their development.

The option of removing the thyroid gland was considered but deliberately rejected by Triplett (1962) as a means of investigating whether interference with the expression of a self-antigen would evoke autoimmunity. Thyroid extirpation was regarded as an inappropriate means of testing this point because, at that time, thyroglobulin was considered to be a 'hidden antigen' to which the immune system was not normally exposed and of which it did not become tolerant. Demonstration of thyroglobulin in the circulation of normal animals disposes of this objection (Daniel *et al.*, 1966). In any event, anti-*thyroid* reactivity should not be considered as synonymous with anti-*thyroglobulin* activity.

The present experiment demonstrates that interference with the development of an organ can render the bearer liable to mount autoimmune responses against tissue from that organ if exposed to it as a result of transplantation in later life. It provides strong experimental support for the proposition that normal development of a tissue, with concurrent expression of its antigenic products, is essential if self-tolerance of those products is to be established.

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