

The influence of the normal microbial flora on the susceptibility of rats to experimental autoimmune thyroiditis

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SUMMARY

Female PVG/c strain rats maintained under specific pathogen-free conditions until weaning were found to be significantly less susceptible to the induction of autoimmune thyroiditis by thymectomy and irradiation than conventionally reared rats of the same strain. Other SPF-derived strains had a similarly low susceptibility, thus pointing to an important external factor influencing the induction of autoimmunity by this procedure. It was found that the oral administration of antibiotics followed by fresh, homogenized, intestinal contents obtained from conventionally reared rats to newly weaned SPF PVG/c rats significantly augmented their autoimmune susceptibility. The offspring of female SPF rats which had been treated in late pregnancy with oral antibiotics and conventional intestinal contents were similarly more susceptible than the offspring of non-treated counterparts. It is proposed that the composition of the normal gastrointestinal flora of conventionally reared rats profoundly influences susceptibility to the induction of autoimmune thyroiditis in this particular experimental model, possibly by antigenic cross-reactivity with thyroid tissue.

Keywords Microbial flora specific pathogen-free rats autoimmune disease thyroiditis thymectomy irradiation

INTRODUCTION

Although a role for infectious agents in the induction of autoimmune disease has been postulated for many years, direct evidence to support this concept is rather limited. Viruses have long been considered as prime suspects in this regard but there is also evidence to suggest that bacteria may provoke autoimmune-mediated tissue injury. For example, the recognition of the association between *Streptococcus pyogenes* group A infection and rheumatic fever is long standing (Kaplan & Meyersian, 1962). Enteric bacteria may also be associated with putative autoimmune conditions. Thus, Ebringer (1979, 1983) has submitted evidence supporting a relationship between the occurrence of ankylosing spondylitis in HLA-B27 positive individuals and the presence of *Klebsiella aerogenes* within the gastrointestinal tract. Similarly, enteric infections caused by *Yersinia enterocolitica* are occasionally complicated by a variety of extra intestinal symptoms such as reactive arthritis, erythema nodosum, iritis and carditis (Winblad, 1968). In addition, an association between thyroid disease and the occurrence of antibodies to *Yersinia* has been reported (Shenkman & Bottone,

1976) and conversely, an increased incidence of antibodies to thyroid epithelium have frequently been noted in patients with *Yersinia* infection (Lidman *et al.*, 1976). Despite these observations, however, consistent evidence of microbial associations with most forms of autoimmune disease is lacking and the role, if any, of these agents in autoimmune aetiology remains to be clarified.

The present investigation was prompted by the observation that PVG/c strain rats which had been reared under specific pathogen-free (SPF) conditions until weaning were markedly less susceptible to the induction of experimental autoimmune thyroiditis by thymectomy and irradiation than conventionally reared rats of the same strain. The SPF and conventional groups were genetically identical being derived from common parental stock originally reared within this institution, thus pointing to an important exogenous factor bearing on autoimmune susceptibility. Since the resident internal microflora of SPF animals, particularly that of the gastrointestinal tract, can be more qualitatively restricted than that of conventionally reared animals it appeared possible that this factor could have been responsible for the differential susceptibility. The chief aim of this investigation was accordingly to explore the hypothesis that elements of the normal gastrointestinal flora influence the induction of autoimmunity in this particular experimental situation.

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MATERIALS AND METHODS

Inbred rat strains. Only a limited number of strains of both SPF and conventional status were locally available for study. SPF female inbred rats of the following strains were obtained from the Animal Resources Centre, Murdoch University, Western Australia; PVG/c, Wistar, LOU/m, BN and WAG. In addition, female PVG/c, Wistar and Lewis rats, bred and reared under conventional conditions, were either obtained from our own animal house or from other local institutions.

Induction of autoimmune thyroiditis. Rats were thymectomized immediately after weaning at 21 days of age by the procedure previously described (Penhale *et al.*, 1973). A series of 4 doses of 25 Gray whole body irradiation at 14 day intervals commencing between 7 and 14 days after thymectomy, were delivered by a Cobalt-60 source (Tosiba, Japan). Rats were irradiated in groups in plastic boxes with sprung lids to restrict movement. Sixty days after the last dose the rats were killed by fluorothane inhalation and blood and tissue samples were taken.

Modification of the gastrointestinal microflora of SPF rats. Two methods were employed for this purpose:

Protocol 1 (Fig. 1): Three-week old female or gonadectomized male SPF PVG/c strain rats were given 30 mg Kanamycin by stomach tube under light fluorothane anaesthesia and the following day 2.5 ml fresh homogenized intestinal contents prepared as described below were similarly administered. The rationale of the antibiotic treatment was to 'decontaminate' the gastrointestinal tract (Van der Waaij *et al.*, 1977) prior to administration of the microflora from a conventionally reared rat.

In each experiment, rats from a limited number of litters thymectomized the previous day were equally divided between 'conventionalized' and control 'SPF' groups. Both groups were given antibiotic treatment but only the conventionalized group received intestinal contents prior to irradiation (Tx-x).

Gonadectomized male rats were utilized as well as female rats as previous studies had shown that gonadectomy elevates male susceptibility to the female level (Penhale & Ansar Ahmed, 1981). Gonadectomy was performed as previously described (Penhale & Ansar Ahmed, 1981) at the time of the microflora administration.

Protocol 2: SPF female PVG/c strain rats in late pregnancy were given 3.5 mg Neomycin and Tetracycline by stomach tube under light fluorothane anaesthesia four times over a period of 7 h. Twenty-four h later 2.5 ml of a fresh suspension of intestinal contents prepared as described below were administered by the same route. This approach was based on the observation that much of the offspring's normal microflora is maternally derived

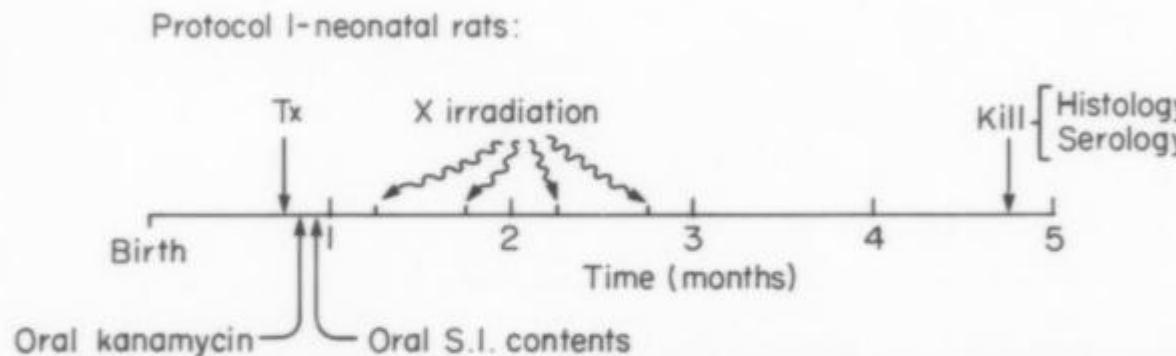


Fig. 1 Protocol 1. Procedure for conversion of SPF rats to 'conventional' status at weaning (3 weeks) and standard thymectomy/irradiation schedule for inducing thyroiditis (S.I. = homogenized gastrointestinal contents).

during the immediate neonatal period (Mackowiak, 1982). Later, the offspring of these conventionalized mothers were subjected to thymectomy and irradiation by the standard schedule without further treatment and their autoimmune parameters compared with those of contemporary offspring. The latter were from SPF mothers given antibiotic treatment alone.

Preparation of gastrointestinal contents. The entire intestinal contents of one conventionally reared Wistar rat were expelled into 10 ml sterile phosphate buffered saline (pH 7.5) at 5°C and gently homogenized. Gross debris was removed by decanting and the remaining suspension administered within 30 min of preparation.

Assessment of thyroiditis. All groups were killed 60 days after final irradiation and the larynx, with thyroid glands *in situ*, fixed in 10% buffered formal saline. Transverse sections taken at three levels were stained with haematoxylin and eosin and examined microscopically for the presence of a mononuclear cell infiltration. The severity of infiltration was visually scored from 0 to 4 according to previously used criteria (Penhale *et al.*, 1973). Assessment was made without prior knowledge of the origin of the sections.

Preparation of rat thyroglobulin (Tg). Approximately 5 g of rat thyroid tissue was suspended in borate buffer pH 8.2 and homogenized mechanically over ice (Polytron, Lucerne). After standing for 30 min at 5°C the homogenate was centrifuged at 30,000 g for 30 min at 5°C. The supernate containing approximately 2 mg/ml protein was subjected to gel permeation chromatography on Sephadex G-25 (Pharmacia, Uppsala) and the fractions containing the exclusion peak were bulked. The concentration of purified Tg was adjusted to 100 µg/ml by ultrafiltration and the solution aliquoted and stored at -30°C.

Measurement of antibody to thyroglobulin. Serum samples were stored at -30°C until required. Antibody titre to thyroglobulin was determined by enzyme immunoassay employing alkaline phosphatase conjugated to affinity purified goat anti-rat IgG (Bio-Rad, Richmond, California).

Round-bottomed 96 well-plates (Sterilin, Feltham, UK) were coated with Tg solution (1 µg/ml) in carbonate/bicarbonate buffer pH 9.6. Serum samples were diluted in Tween-PBS and following incubation with conjugate and substrate their optical density was measured in a multichannel spectrophotometer.

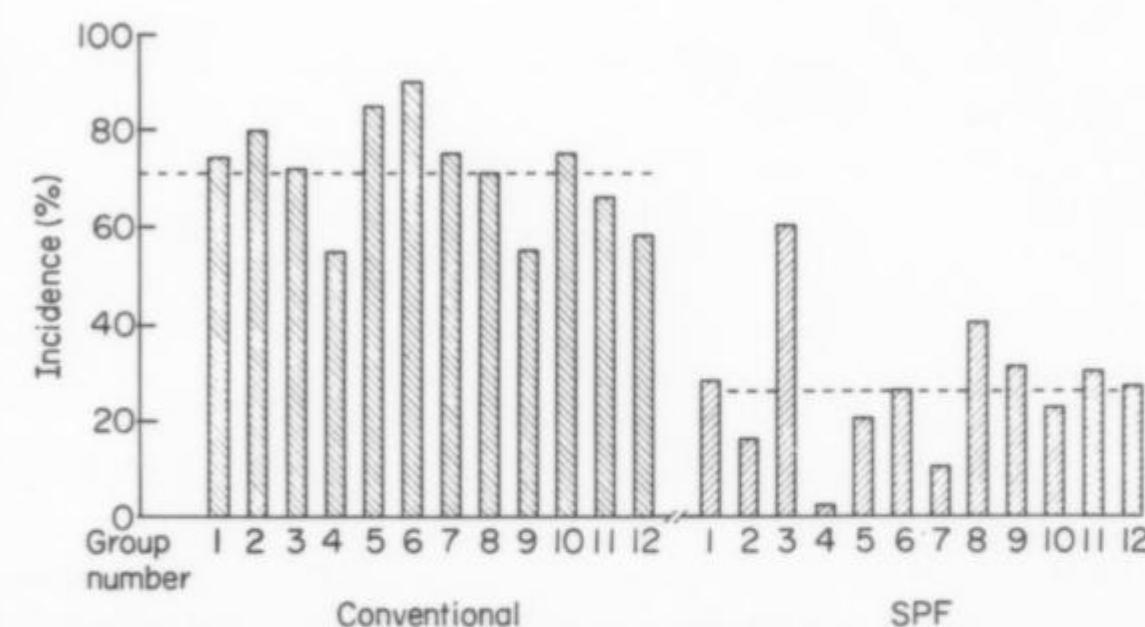


Fig. 2 The incidence of thyroiditis (i.e. pathology score of 1 or greater) in sequential groups of thymectomized and irradiated rats of conventional and SPF status. Each group contained at least 10 female rats.

Table 1. The occurrence of autoimmunity in conventional vs SPF rats of different inbred strains

Strain	Conventionalized						SPF					
	No. of Rats*	Thyroid pathology		Tg autoantibody		No. of Rats	Thyroid pathology		Tg autoantibody			
		Incidence (%)	Mean index ± s.e.m.	Incidence (%)	Mean titre ± s.e.m. (\log_2)		Incidence (%)	Mean index ± s.e.m.	Incidence (%)	Mean titre ± s.e.m. (\log_2)		
PVG/c†	16	100·0	2·8 ± 0·1	100·0	10·3 ± 0·6	51	15·6	0·2 ± 0·1	25·4	1·1 ± 0·3		
Wistar†	14	50·0	1·1 ± 0·3	42·0	2·2 ± 0·8	6	0	0	0	0		
Lewis	5	20·0	0·8 ± 0·8	60·0	3·2 ± 1·5	—	—	—	—	—		
Lou/m	—	—	—	—	—	11	0	0	0	0		
BN	—	—	—	—	—	6	16·0	0·2 ± 0·2	0	0		
WAG	—	—	—	—	—	9	11·0	0·1 ± 0·1	0	0		
All†	35	68·8	1·8 ± 0·2	68·8	6·1 ± 0·8	83	12·0	0·1 ± 0·1	15·6	0·7 ± 0·2		

* Female rats subjects to thymectomy and irradiation.

† Statistical analysis: Incidences (χ^2)—Thyroid pathology: PVG/c $P < 0·001$, Wistar $P < 0·05$, All groups $P < 0·001$. Tg autoantibody: PVG/c $P < 0·001$, Wistar $P < 0·05$, All groups $P < 0·001$.

meter (Titertek Multiskan, Flow Laboratories). The reciprocal of the highest dilution giving a reading twice background was taken as the titre of the sample.

RESULTS

The incidence of thyroiditis in sequential groups of SPF and conventional thymectomized and irradiated PVG/c strain rats. Incidence data from successive groups of Tx-x treated conventional and SPF rats established over a period of approximately 36 months are shown in Fig. 2. Conventionally reared groups of identical genetic background had a consistently higher incidence (mean 71%) of thyroiditis (i.e. a pathology score of 1 or greater) than the SPF (mean 20%). This differential, highly significant by χ^2 analysis ($P < 0·001$), was maintained throughout the period of investigation. However, considerable variation was observed between groups within each status over this period. Thus

incidence ranged from 55 to 90% in the conventional and from 2 to 60% within the SPF series. In the latter groups, only one (group 3), had an incidence level (60%) similar to those of the conventional series. The incidence of autoantibodies to Tg also paralleled this pattern throughout the series (results not shown).

Since the SPF-derived and conventional rats were genetically identical these observations pointed to a significant environmental contribution to autoimmune susceptibility.

The incidence of autoimmunity in conventional versus SPF rats of various strains following thymectomy and irradiation. In order to determine whether the above observation applied to all conventionally reared rats regardless of strain both conventional and SPF female rats of a number of readily available inbred strains were subjected to thymectomy and irradiation. The incidence of thyroiditis and autoantibodies subsequently observed is shown in Table 1. The conventionally reared rats of both PVG/c and Wistar strains had a significantly greater

Table 2. The occurrence of autoimmunity following conversion of SPF rats to conventional status at weaning

Expt No.	No. of Rats*	Conventionalized						SPF					
		Thyroid pathology		Tg autoantibody		No. of Rats	Thyroid pathology		Tg autoantibody				
		Incidence (%)	Mean index ± s.e.m.	Incidence (%)	Mean titre ± s.e.m. (\log_2)		Incidence (%)	Mean index ± s.e.m.	Incidence (%)	Mean titre ± s.e.m. (\log_2)			
1	7	28·4	0·4 ± 0·3	56·8	2·6 ± 1·0	4	0	0	0	0			
2	6	16·6	0·3 ± 0·1	66·7	3·2 ± 0·5	6	0	0	0	0			
3	6	50·0	1·2 ± 0·5	83·0	5·1 ± 1·4	12	41·7	1·0 ± 0·1	50·0	2·7 ± 0·3			
4	8	38·0	1·1 ± 0·5	37·5	1·8 ± 0·9	9	33·0	0·9 ± 0·2	22·0	1·6 ± 0·5			
5	8	75·0	2·0 ± 0·5	75·0	4·1 ± 1·2	10	40·0	0·9 ± 0·1	27·0	1·7 ± 0·4			
All†	35	42·8	1·2 ± 0·2	62·8	3·0 ± 0·5	41	29·3	0·7 ± 0·2	31·7	1·4 ± 0·5			

* Female only Expts 1, 2 and 5. Equal numbers of females and gonadectomized males Expts 3 and 4.

† Statistical analysis: Incidences (χ^2)—Thyroid pathology: $P < 0·05$, Tg autoantibody: $P < 0·01$. Levels (Student's *t*-test)—Thyroid pathology: $P > 0·05$, Tg autoantibody: $P < 0·05$. Each expt included groups of SPF rats of PVG/c strain from one or more litters which were divided into 'conventionalized' (i.e. treated with antibiotic followed by intestinal material) or 'SPF' (i.e. antibiotic treated only) before thymectomy and irradiation.

Table 3. The occurrence of autoimmunity in the offspring of female SPF rats converted to conventional status prior to parturition

Expt No.	Conventionalized						SPF					
	No. Rats	Sex	Thyroid pathology		Tg autoantibody		No. of Rats	Sex	Thyroid pathology		Tg autoantibody	
			Incidence (%)	Mean index \pm s.e.m.	Incidence (%)	Mean titre \pm s.e.m. (\log_2)			Incidence (%)	Mean index \pm s.e.m.	Incidence (%)	Mean titre \pm s.e.m. (\log_2)
1	4	♂	25·0	0·5 \pm 0·5	75·0	3·8 \pm 1·3	4	♂	25·0	0·5 \pm 0·5	25·0	1·7 \pm 1·7
	7	♀	57·0	1·3 \pm 0·6	71·4	5·1 \pm 1·7		♀	14·2	0·1 \pm 0·1	14·2	0·7 \pm 0·8
2	5	♀	60·0	1·6 \pm 0·8	60·0	5·6 \pm 2·5	3	♀	0	0	0	0
3	3	♀	NA*	NA	100·0	6·3 \pm 0·7	6	♀	NA	NA	33·0	2·7 \pm 1·7
All†	19		50·0	1·2 \pm 0·4	73·7	5·2 \pm 0·9	20		14·3	0·2 \pm 0·2	20·0	1·4 \pm 0·7

* NA—not available.

† Statistical analysis (♀ only): Incidences (χ^2)—Thyroid pathology: $P < 0·05$, Tg autoantibody: $P < 0·001$. Levels (Student's *t*-test)—Thyroid pathology: $P < 0·05$, Tg autoantibody: $P < 0·001$. In each expt a litter from an SPF female converted to conventional status and a litter from an untreated SPF female were subjected to thymectomy and irradiation.

incidence of lesions and autoantibody than their SPF counterparts. The extent of thyroid infiltration and Tg autoantibody titres were also correspondingly greater in the conventionally reared rats. Overall, these findings suggested that the conventional groups were strongly influenced by an external factor which operated independently of genetic background.

Thyroid autoimmunity in SPF rats converted to conventional strains at weaning. In order to investigate the possibility of a microfloral influence, fresh intestinal contents obtained from healthy conventionally reared rats were administered to antibiotic-treated weaner SPF PVG/c strain rats prior to thymectomy and irradiation (Protocol 1). The results of a series of such experiments are shown in Table 2.

Incidence and severity of thyroid change were consistently greater in the converted as compared to untreated SPF littermates. Autoantibody data also followed a similar pattern. Although serial experiments performed over a period of approximately 5 months were essentially similar in this regard, considerable inter-group variation was noted particularly between early and late experiments.

Thyroid autoimmunity in the thymectomized and irradiated offspring of female SPF rats converted to conventional status. The results of three experiments in which thymectomy and irradiation were performed on the offspring of mothers to which intestinal contents derived from conventionally reared rats had been administered prior to parturition (Protocol 2) are shown in Table 3. Overall, with the exception of the entire male offspring in Expt 1, groups born of treated mothers were markedly more susceptible to the induction procedure than those of untreated mothers. This effect was most striking in Expt 2 where autoimmune change was extensive in offspring of the treated mothers and absent in the untreated.

DISCUSSION

The observations presented above indicate that, regardless of genetic background, exogenous environmental factors can exert a significant influence on susceptibility to experimental thyroid autoimmunity in rats induced by thymectomy and irradiation. In addition, evidence has been obtained to support the concept

that the major environmental factor concerned is the normal intestinal microflora although the involvement of additional extrinsic factors is not excluded. Thus the low susceptibility of SPF rats could be augmented by administering fresh intestinal material derived from conventionally reared animals either directly at weaning or indirectly via pregnant females. Although not directly established, it is assumed that these procedures lead to the transfer and establishment of a more extensive range of micro-organisms than that normally present in the microflora of SPF animals. This inference would seem likely since the time scale of these experiments (at least 90 days between treatment and termination) would appear to preclude any short-term non-replicating influence. Furthermore, in Protocol 2 no direct treatment whatsoever was given to the experimental group.

The significance of this influence with respect to triggering as opposed to the modulation of autoimmunity was demonstrated by the observation that in some groups, disease could only be induced following treatment, indicating that the effect is exerted at the level of the primary inductive event. It was found unnecessary to maintain mature SPF rats in special isolation conditions in order to preserve their lower susceptibility status and the SPF groups were kept in close proximity (adjacent rooms) to the conventional animals throughout these experiments. However, the considerable variation in incidence noted, particularly between early and late groups in serial experiments, might be explained by difference in background levels of relevant microbial flora.

There are a number of possible mechanisms whereby the intestinal microflora could be involved in raising susceptibility to auto-immunity. These include: (1) polyclonal activation of B lymphocytes by microbial products such as the cell wall lipopolysaccharide of gram-negative bacteria which can bypass the requirement for T-cell help (Esquivel, Rose & Kong, 1977; Izui, Eisenberg & Dixon, 1979); (2) Microbial stimulation of local cellular expression of class II histocompatibility molecules thereby initiating the antigenic presentation of cell-membrane-located molecules to appropriate T cells (Bottazzo, Pujol-Borrell & Hanafusa, 1983); (3) Microbial modification of body constituents either by direct combination or enzymatically thus rendering these immunogenic (Geczy *et al.*, 1983); or (4)

Mimicry of potentially autoantigenic self-molecules by microbial components causing immunological cross-reactivity (Kaplan & Meyserian, 1962). Of these possibilities the last is appealing since cross-reactivity between infectious agents and body constituents is now a widely recognized biological phenomenon. For example, cross-reactivity has been demonstrated between bacterial products and a broad range of structural and soluble body components including histocompatibility and blood group antigens (Springer, 1971; Ebringer, 1979) hormones and their cell receptors (Marcio *et al.*, 1979; Le Roith *et al.*, 1981; Stefansson *et al.*, 1985) together with a range of cytoplasmic and nuclear constituents (Schoenfeld *et al.*, 1983).

The microbial origin of an antigenic molecule resembling a potentially autoantigenic thyroid component would explain the thyroid-restricted nature of the autoimmune manifestations observed in this particular model (Penhale *et al.*, 1973). Moreover, in contrast to endogenously derived self-antigens which are usually self-tolerizing, microbially derived epitopes are likely to be appropriately processed and presented to T cells in association with MHC class II products on the cell membrane of antigen-presenting cells.

In the context of microbial cross-reactivity and disease pathogenesis it can be envisaged that the effect of the experimental manipulation is 2-fold; first, it may depress regulatory function (Penhale *et al.*, 1976) and second, it may promote the influx of cross-reactive antigen as a consequence of irradiation-induced damage to enterocytes. Thus an appropriate combination of events may be required to initiate autoimmunity: (1) the presence within the gastrointestinal tract of a micro-organism producing a cross-reactive epitope; (2) the passage of this epitope across the intestinal barrier and finally (3) depressed immune regulatory function. If applicable to clinical disease such an exacting combination of requirements could account for the characteristic onset and incidence pattern of this type of disease.

In conclusion, this study presents evidence of a significant exogenous contribution to the inductive process of an experimental model of organ-specific autoimmune disease. The ability to manipulate this model by the procedures described opens the prospect of further studies which may shed light on the aetiology of this important form of autoimmune disease.

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