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Gut flora induces and maintains resistance against streptococcal cell wall-induced arthritis in F344 rats

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SUMMARY

Streptococcal cell wall (SCW)-induced arthritis is a chronic, erosive polyarthritis that can be induced in susceptible Lewis rats by one i.p. injection of an aqueous, sterile suspension of SCW. F344 rats are resistant to chronic joint inflammation. Our previous studies showed a correlation between susceptibility to SCW-induced arthritis and the ability to mount SCW-specific T cell responses, suggesting tolerance to SCW as a putative mechanism. Here we prevented the induction of tolerance to bacterial epitopes in F344 rats by using them germ-free and analysed susceptibility to arthritis subsequently. In addition, we conventionalized germ-free F344 rats at different times before induction of arthritis. Our results show that germ-free F344 rats are susceptible to SCW-induced arthritis with a similar severity, chronicity, incidence and onset as Lewis rats. Moreover, T cells isolated from germ-free F344 rats were able to respond to SCW. Conventionalization dramatically moderates arthritis and makes T cells unresponsive to SCW again. Thus, in normal rats (F344) a state of tolerance to arthritogenic epitopes is induced (neonatally) and maintained through life by the bacterial flora, resulting in resistance to bacterium-induced artritides. In arthritis-prone (Lewis) rats, this tolerance is deficient and/or easily broken.

Keywords streptococcal cell wall arthritis gut flora resistance

INTRODUCTION

Streptococcal cell wall (SCW)-induced arthritis is a chronic, erosive polyarthritis which can be induced in susceptible Lewis rats by a single i.p. injection of a sterile, aqueous suspension of SCW [1]. The acute phase of the disease develops within a few days and is dependent on systemic activation of complement [2]; the second, chronic phase develops from day 10 onwards. Dependent on the SCW batch used, either biphasic disease or only the chronic phase may be seen. Thymectomized [3,4], cyclosporin A-treated [4,5] or nude Lewis rats [6], as well as F344 rats [7] are resistant to the second, destructive episode of inflammation, whereas they undergo the acute phase with comparable severity to the Lewis rat. Together, these data strongly suggest an absolute dependence on functional T lymphocytes of the chronic phase of SCW-induced arthritis. An ultimate proof for the crucial involvement of T cells and even a hint to SCW-induced arthritis being an autoimmune disease came from adoptive transfer experiments in SCW-induced arthritis [8]. In addition, we have described a correlation between susceptibility to chronic SCW-induced arthritis and the ability to mount SCW-specific T cell responses [9,10]. Prevention of the development of SCW-specific T cells in Lewis rats, for instance by pretreatment with the mycobacterial 65-kD heat shock protein [11] or with small amounts of cell walls (unpublished results), results in resistance to SCW-induced arthritis. Moreover, treatment of Lewis rats with MoAbs against CD4 makes Lewis rats completely refractory to SCW-induced arthritis [20] and induces a long-term tolerance to SCW, coinciding with long-term resistance to SCW-induced arthritis.

These results suggested that T cell unresponsiveness due to immunological tolerance to SCW may be the mechanism underlying resistance to SCW-induced arthritis. At the same time, we assume that Lewis rats are defective in their tolerance, which fits the observation that Lewis rats are susceptible to almost every inducible model for autoimmunity. To study this hypothesis, we tried to prevent the induction of tolerance to (arthritogenic) bacterial antigens in F344 rats to see whether this manipulation would result in susceptibility. Because all bacteria (environment, gut) may induce tolerance to certain (arthritogenic) epitopes immediately after birth, we used germ-free F344 rats for the induction of SCW arthritis and analysis of SCW-specific T cell responses.

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MATERIALS AND METHODS

Rats

Germ-free (GF) F344 rats were obtained originally from the Jackson Laboratories (Bar Harbor, ME) and were bred in overpressure isolators in our own facilities. The isolators and faeces were screened once every two weeks for bacterial contamination. Animals in contaminated isolators were never used for experiments. Conventional (CV) F344 rats were obtained from Jackson Laboratories. CV Lewis rats were originally obtained from the Zentral Institut für Versuchstierzucht (Hannover, Germany) and bred in our own facilities.

Female rats weighing 130–160 g were used for the experiments. Rats were fed tap water and standard chow, which was sterilized in case of GF rats.

Conventionalization of GF rats

GF F344 rats were removed from the isolater and were put into a cage in which also a CV F344 was housed. One group was moved to a so-called 'dirty cage' 6 weeks prior to induction of arthritis and another group was moved 3 weeks prior to induction. Comparable experiments in mice demonstrated that conventionalization takes place within 12 days [12,13].

Streptococcal cell walls

Streptococcus pyogenes (group A) organisms were grown in Todd Hewitt broth to late log/early stationary phase, harvested and disrupted mechanically by shaking with glass beads. The effectivity of this treatment was checked by Gram staining. The fragments were subsequently treated with RNAse, DNAse and trypsin as described [1]. Further isolation was carried out by differential ultracentrifugation steps as described [14] and the 100 000 ***g*** pellet was used after ultrasonic treatment throughout the experiments. This preparation contained approximately 12% muramic acid [15].

Induction of SCW arthritis

To induce a chronic polyarthritis, rats were injected intraperitoneally with a sterile, aqueous suspension of SCW at a dose of 22 μg muramic acid/1 g rat body weight. The arthritis was scored macroscopically by measuring paw thickness with a caliper and by histology.

Proliferative response of spleen cells

Spleens were removed aseptically from the rats 21–35 days after i.p. SCW injection and a single-cell suspension was made. Erythrocytes were lysed (0·16 M NH_4Cl/0·17 M Tris–HCl, pH 7·2) and the leucocytes were washed twice with IMDM (Flow Laboratories). Adherent cells were removed by incubating the cells in a plastic culture flask (Costar) in IMDM, containing 2% SF-1 (Costar) as a serum substitute at a cell density of 5×10^6/ml. After 1 h in a CO_2-incubator, non-adherent cells were aspirated and pooled per group (eight for CV rats group; five for GF rats group; and four for conventionalized rats group). Cells were put into culture with a final density of 1×10^6/ml in 0·2 ml volumes of IMDM + 2% SF-1 + 10 mM pyruvate + 20 mM glutamine + 40 μg/ml gentamycin + 5 μM β-mercaptoethanol. Cells were cultured in 96-well round-bottomed plates (Costar) for 3 days with concanavalin A (1–0·5 μg/ml) or with SCW (6·2–0·6 μg/ml muramic acid). Subsequently, 37 kBq ^{3}H-thymidine was added per well and cells were harvested after 24 h.

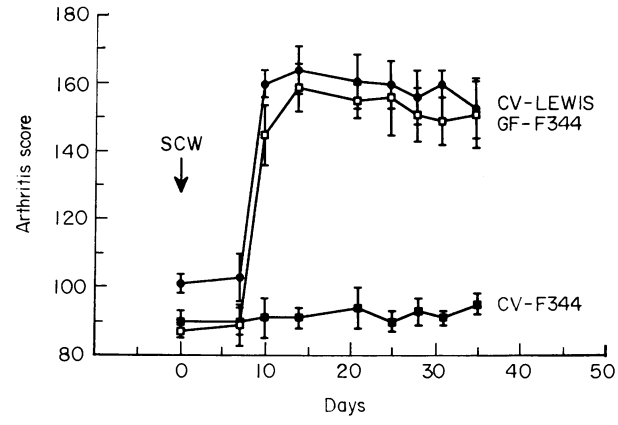

Fig. 1. Effect of gut flora on susceptibility to SCW-induced arthritis. Arthritis is induced at day 0 by injecting SCW intraperitoneally. Arthritis is expressed as the sum of the thickness of both hind-paws (×0·1 mm). Values are the mean ± s.d. from groups of five to eight rats. CV, conventional; GF, germ free.

Table 1. Histological score of streptococcal cell wall-induced arthritis

		Rats (*n*) with a score of			
Rats	*n*	0	1	2	3
CV-L	10	0	1	8	1
CV-F	8	8	0	0	0
GF-F	6	0	2	4	0
GF-F/−3	8	1	4	3	0
FG-F/−6	4	2	2	0	0

Ankles were prepared for histology (haematoxylin & eosin staining) 35 days after induction of arthritis. Arthritis was scored using a scale of 0–3 by two independent observers on coded sections: 0, no sign of inflammation; 1, 2, 3, inflammatory mass (infiltrate, exudate) comprises <25%, 25–50%, >50% of joint, respectively.

CV, conventional; GF, germ free; F, F344; L, Lewis; /−3, −6 conventionalized at 3 and 6 weeks, respectively, before arthritis induction.

Proliferation is expressed as stimulation index: ct/min due to stimulus X divided by ct/min due to medium alone. All values represent the mean of at least triplicate cultures and s.d. was always within 10%.

Histology of ankle joints

Ankle joints were removed *in toto* 21–35 days after i.p. injection with SCW and processed for histology as described [16] with few alterations: fixation in formalin for 2 weeks and decalcification for 2 weeks. Paraffin sections (7 μM) were stained with haematoxylin and eosin. Scoring of inflammation was done by two independent observers on coded slides. A scale of 0–3 was used to quantify the amount of infiltrate and exudate.

RESULTS

Comparison of arthritis in GF F344, CV F344 and CV Lewis rats

Arthritis was induced at day 0 in CV Lewis (positive control) CV F344 rats (negative control) and in GF F344 rats. Thickne

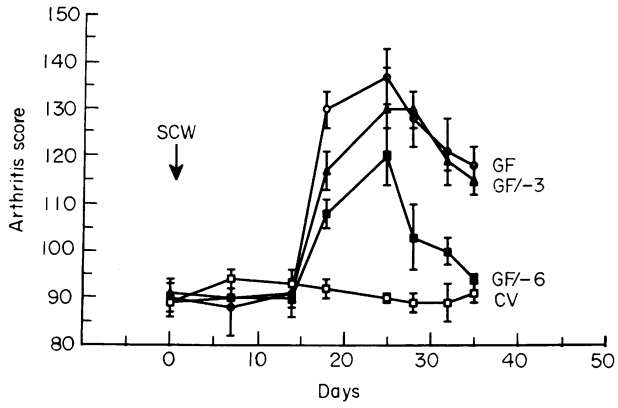

Fig. 2. Effect of conventionalization of F344 rats on susceptibility to SCW-induced arthritis. Arthritis is induced at day 0 by injecting SCW intraperitoneally. Arthritis is expressed as the sum of the thickness of both hind-paws ($\times 0{\cdot}1$ mm). Values are the mean $\pm$ s.d. from groups of four to eight rats. CV, conventional; GF, germ free; GF/−3, GF/−6, germ-free F344 rats conventionalized 3 and 6 weeks before arthritis induction, respectively.

of the hind-paws was measured in all groups (five to eight rats per group) and the mean values $\pm$ s.d. of a representative experiment are given in Fig. 1. SCW-arthritis resistant CV F344 rats do not develop the chronic, erosive polyarthritis upon injection with SCW, while susceptible CV Lewis rats do. These data are in agreement with our own and others' previous data [1,4,7–11]. However, the GF F344 rats develop chronic polyarthritis to an extent similar to Lewis rats, regarding onset, vehemence, incidence and chronicity.

The above mentioned data were confirmed histologically (Table 1).

The effect of conventionalization of GF F344 rats on the susceptibility to SCW-induced arthritis

SCW were injected in GF and in CV F344 rats and additionally in GF F344 rats which were removed from the sterile isolator and were housed subsequently in a dirty cage together with a CV F344 rat at 6 (GF/−6) or at 3 (GF/−3) weeks before induction of arthritis. Again, CV F344 rats are resistant, and GF F344 rats are susceptible (Fig. 2, Table 1) to SCW-induced arthritis. GF/−3 rats are as susceptible as GF rats, while GF/−6 rats also develop an arthritis, but with a slightly later onset, a lower maximum paw swelling and a significantly earlier decrease than GF F344 rats. These results show that tolerance to bacteria and resistance to SCW-induced arthritis are causally related and that this tolerance can be induced in adult rats. In addition, one may conclude that tolerance takes less than 6 weeks to develop, and T cell responses suggest (Table 2b) that tolerance is detectable even earlier than 3 weeks after conventionalization.

Proliferative response of splenic non-adherent cells (SNAC)

To investigate the mechanism underlying the different susceptibility to SCW-induced arthritis, we analysed T cell responses to SCW at the end of each experiment (30–35 days after SCW injection). Comparison of these responses in CV F344, CV Lewis and GF F344 rats (Table 2) suggested a correlation between susceptibility and presence of SCW-responsive T cells in the spleen.

If we screened GF, CV and conventionalized (GF/−3 and GF/−6) F344 rats with respect to SCW-specific T cell responses, the same correlation was found (Table 2): SNAC isolated from GF (susceptible) F344 rats proliferated to SCW, whereas SNAC isolated from CV (resistant) F344 rats did not. SNAC from conventionalized F344 rats displayed an intermediate response to SCW: if conventionalization was established 3 weeks prior to arthritis induction, rats still were susceptible (Fig 2), and their T cells responded to SCW *in vitro*. If conventionalization was carried out 6 weeks before arthritis induction, rats were slightly susceptible, and their T cells did not respond to SCW *in vitro*.

Thus, induction of a T cell non-responsiveness to SCW (or to bacterial antigens in general) results in resistance to SCW arthritis.

DISCUSSION

Chronic, erosive polyarthritis induced by i.p. administration of a single dose of SCW in an aqueous, sterile suspension develops easily in susceptible Lewis rats, whereas most other strains are resistant [1,2,7]. The fact that F344 rats are resistant excludes that MHC class I and/or II antigens, and thus epitope selection, are of decisive importance. However, functional T lymphocytes have been shown to play a pivotal role in susceptibility to SCW-induced, chronic joint inflammation; there even is evidence that SCW-specific T cells are required [3–6,8–11,17]. Thus, an answer to the question why T cells from F344 rats do not respond to SCW after priming with cell walls might elucidate the mechanism of resistance to SCW arthritis.

Induction of T cell unresponsiveness to SCW in Lewis rats by pretreatment with low-dose cell walls (unpublished data) or with the 65-kD bacterial common antigen [11] leads to resistance to SCW-induced arthritis in the same rats. In addition, another well-known means to induce tolerance, priming when $CD4^+$ cells are depleted [18,19], also leads to long-term resistance to SCW-arthritis in Lewis rats [20]. Together, these data suggest immunological tolerance to SCW as a mechanism for resistance to SCW arthritis.

Our results support our hypothesis on tolerance: GF F344 rats in which tolerance to bacteria could not have developed, because they never encountered bacteria, are as susceptible to SCW-induced arthritis as are Lewis rats. That tolerance indeed can be, and probably is induced by bacteria in F344 rats, is shown in conventionalization experiments: contact between the immune system of adult rats and bacteria induces T cell tolerance to SCW and resistance to SCW arthritis within 6 weeks. When the contact lasted 2–3 weeks or less, the tolerance and thus resistance was obvious although not complete.

Our findings are concordant with those described for adjuvant arthritis [21,22], a model with a pattern of susceptibility which is similar to that observed in SCW arthritis. Monoassociation of GF F344 rats with Gram-negative *Escherichia coli* resulted in resistance which equalled that in CV F344 rats whereas monoassociation with Gram-positive *Lactobacillus casei* did not really affect susceptibility. A combination of both bacteria suppressed susceptibility to a similar extent as *E. coli* alone. This suggests that Gram-negative bacterium-specific compounds, e.g. lipopolysaccharide (LPS; a known immunomodulator [23,24]), are responsible for decreased susceptibility.

Table 2. Proliferative response of splenic non-adherent cells

	Stimulation index			Stimulation index			
Stimulus (μg/ml)	CV-L	CV-F	GF-F	GF-F	GF-F/−3	GF-F/−6	CV-F
SCW							
(6)*	25	3	19	10	5	2	2
(2)	12	2	9	6	4	1	1
(0·6)	6	1	4	—	—	—	—
Con A (1)	104	24	84	29	26	25	26
ct/min on medium	1993	2137	1589	970	1171	1439	1396

Stimulation index = ct/min with stimulus/ct/min without.

* Streptococcal cell wall (SCW) expressed in muramic acid equivalents.

Non-adherent cells were isolated from the spleen 35 days after arthritis induction. One-hundred thousand cells per well were incubated with various stimuli and proliferation was determined by the incorporation of ^{3}H-thymidine from day 3 to day 4 of culture. Results are the mean of triplicate cultures, and s.d. was always < 10%.

Because we did not make gnotobiotic rats but conventionalized with total F344 flora, we can't define the molecule(s) responsible for the induced resistance. Of course, LPS is a plausible candidate, but also peptidoglycans are known to have immunomodulatory capacities [25]. Because our lymphocyte stimulation data suggest antigen-specific tolerance, peptidoglycans are likelier to be the 'tolerogen'. Moreover, injection of 50 μg LPS 2 weeks before induction of SCW-arthritis has no influence on the development of arthritis in susceptible Lewis rats (unpublished observation).

Having suggested immunological tolerance to (arthritogenic) bacterial epitopes as a protective mechanism against bacterium-induced arthritides (SCW-induced and adjuvant arthritis) we automatically imply a defective tolerance to similar epitopes in susceptible Lewis rats. This is at least not contradictory to the observations that Lewis rats are extremely susceptible, and sometimes even uniquely susceptible, to inducible models of autoimmunity involving various target organs.

We think that in 'normal' rats, like the F344 rat, the immune system protects itself (neonatally) against disease-inducing responses by immunological non-responsiveness, whereas in autoimmunity-prone rats, like the Lewis rat, this protective mechanism is absent or easily overruled by strong stimuli. In Lewis rats, resistance to various autoimmune diseases can be achieved by tolerization with relevant antigens and/or epitopes. This induced tolerance, however, unlike neonatally induced tolerance, is not life long.

In addition, in bacterium-induced polyarthritis, as in most other models for autoimmunity, a permissive MHC class I and/or II seems to be obligatory, because resistant Wistar rats do not become susceptible to cell wall or adjuvant arthritis when tested germfree (Peter Heidt, TNO, Rijswijk, The Netherlands, personal communication).

The significance of these findings for human disease from a therapeutical point of view is unclear at the moment, because we did not interfere with ongoing disease in this report. Additionally, it has never been proven in man that autoimmunity results from loss of tolerance. However, theoretically, tolerization of arthritic patients for target antigens may lead to reduction of inflammation.

The way by which tolerance is maintained in CV F344 rats and in GF F344 rats after conventionalization is at the moment the subject of further investigations in our department.

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