Prevention of adjuvant arthritis in Lewis rats by neonatal bacille Calmette–Guérin (BCG) infection

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SUMMARY

Tolerization of pathogenic antigens is one of the experimental strategies that has been proposed to prevent autoimmune disease. We have investigated here whether neonatal intraperitoneal infection of Lewis rats with *Mycobacterium bovis*-BCG has any effect on the expression of adjuvant arthritis (AA), an autoimmune disease that is produced by immunization of the rats with dead mycobacteria in mineral oil (i.e. Freund's complete adjuvant (FCA)). We found that neonatal infection with 10^8 viable BCG bacilli rendered all Lewis rats resistant to the expression of AA after FCA immunization. This BCG-induced protection from reactive arthritis was not seen in Lewis rats infected with smaller inocula (10^6 BCG bacilli) or if the infection was performed after the neonatal period (e.g. at 3 weeks of age). Neonatal administration of 65-kD mycobacterial heat shock protein (hsp65, a key antigen in the etiopathogenesis of AA) failed to protect Lewis rats from AA; injection of lactoferrin (an autoantigen that may be involved in the physiopathology of autoimmune arthritis) to newborn Lewis rats decreased the severity of AA observed after FCA immunization of the animals. Western blotting revealed that Lewis rats that had acquired resistance to AA also showed changes in their repertoire of antibody specificities; among these alterations was decreased anti-hsp65 reactivity. We conclude that neonatal infection with BCG, but not hsp65 injection, renders Lewis rats resistant to AA and that the phenomenon is associated with change in the repertoire of specificities of circulating antibodies.

Keywords autoimmunity antibodies rheumatoid arthritis *Mycobacterium bovis*-BCG mycobacteria

INTRODUCTION

Infection is an important environmental factor of autoimmune disease [1,2]. Mycobacteria are among the infectious agents that have been found, both in humans and in experimental models, to be able to modulate autoimmune disease, namely by triggering [1–4], aggravating [5,6], or even preventing [7,8] autoaggressive disorders.

The classical experimental model of mycobacteria-induced autoimmune disease is adjuvant arthritis (AA) [3,4,9], which is produced by a single immunization of Lewis rats with a variant of Freund's complete adjuvant (FCA; i.e. heat-killed mycobacteria in suspension in mineral oil). AA is expressed by an inflammatory reaction of the sinovial tissue that is observed 2–3 weeks after the FCA injection; the disease leads to distortion of the joints resembling human autoimmune arthritis [3,4,9]. After 6–8 weeks, the acute inflammation subsides but deformation of the paws is still found [3,4,9].

Here, we have used the AA model to investigate whether neonatal immunization of Lewis rats with *Mycobacteria bovis*-BCG changes the expression of FCA-induced AA triggered in adult rats. We chose *Myco. bovis*-BCG for two reasons: (i) this mycobacterial species is massively used in vaccination of children in numerous developing countries where tuberculosis is endemic; and (ii) there has been speculation on whether BCG vaccination is able to modify the susceptibility of the host to autoimmune arthritis [10].

We compare here the incidence of AA in Lewis rats that were submitted to neonatal inoculation with BCG with the incidence of AA in control Lewis rats and in rats neonatally injected with AA-related antigens. In addition, we have used Western blotting to search for changes in the repertoire of antibody specificities in the Lewis rats submitted to the different types of neonatal immunizations. We document that neonatal BCG inoculation renders adult Lewis rats resistant to AA and that it also modifies the specificity of circulating antibodies that recognize mycobacterial antigens. Portions of this work were presented before in abstract form [11].

MATERIALS AND METHODS

Rats
Parental inbred Lewis rats were supplied by Harlan Olac Ltd (Bicester, UK). The animals were kept in sterile quarters and had free access to food and water.

Mycobacteria
Mycobacterium bovis-BCG (TMCC 1011, strain Pasteur) was grown in liquid culture using Midlebrook 7H9 broth (Difco Labs, Detroit, MI) containing 0·04% Tween 80. Mycobacteria were harvested from liquid culture by centrifugation (6000 g) and washed three times in PBS. The bacteria were suspended in saline containing 0·04% Tween 80 and diluted to a concentration of 10^8 viable bacilli of *Myco. bovis*-BCG per ml.

Antigens
Heat-killed *Myco. tuberculosis* H37Ra was obtained from Difco Labs. The mycobacterial 65-kD heat shock protein (hsp65) was produced in Dr Jan D. A. van Embden's Laboratory (National Institute of Public Health and Environmental Sciences, Bilthoven, The Netherlands); it is the *Myco. bovis*-BCG hsp65 element cloned and expressed in *Escherichia coli* as described by Thole *et al.* [12]. Human lactoferrin (LF) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO).

Immunizations
Seventy-six Lewis rats of both sexes were used in this study. The rats were divided into 10 groups of 6–12 animals that were submitted to i.p. injection of different inocula at the neonatal period, that is less than 24 h after the birth of the rats. The neonatal inocula were the following: 10^6 or 10^8 viable *Myco. bovis*-BCG bacilli, 50 μg of either hsp65, LF or BSA. Hsp65 is a key antigen in the etiopathogenesis of AA [9,13]; LF has also been implicated in mycobacteria-induced autoimmunity [14–18]; BSA was used as a control antigen, i.e. an antigen not involved in the triggering of AA. All inocula were made in 500 ml of saline buffer; control rats were neonatally injected with 500 ml of saline in the peritoneal cavity. Additional groups of rats were submitted to i.p. injection of 10^8 viable *Myco. bovis*-BCG bacilli after the neonatal period had ended (in our experiments, at 3 weeks of age).

In order to determine the effect of the neonatal treatments on the incidence of FCA-induced arthritis in adult Lewis rats, all rats were subsequently, i.e. when they were 8 weeks old, immunized with the arthritogenic FCA emulsion. Our preparation of FCA was obtained by thorough crushing of dead *Myco. tuberculosis* H37Ra bacilli into a fine powder (using a mortar and a pestle) before mixing with mineral oil, as previously described [9]; 100 ml of the preparation were intradermally injected at the base of the tail. The rats were examined during 6 weeks for signs of arthritis. The rats were killed when they were 14 weeks old and sera were collected for the immunoblotting studies.

Incidence and severity of arthritis
In order to determine the incidence of arthritis in the different groups of Lewis rats, the animals were examined three times a week during the 6 weeks that followed the arthritogenic FCA injection. Lewis rats were considered to show arthritis if both swelling and reddening of joints of at least two limbs were observed for 2 weeks or more.

To evaluate the severity of arthritis in individual rats we adopted a scoring method that has been used by other authors [9,13]. This method is based on naked eye examination of the four limbs of the rats in order to assign 0–4 score of arthritis severity to each limb. The arthritis score for individual rats was calculated by adding the scores given to each of the four limbs; thus, the maximum score for arthritis severity that can be assigned to a single rat is 16. Grade 0 corresponded to animals with no sign of joint inflammation; grade 1 indicated that the joints showed some swelling and redness; grade 2 indicated moderate swelling and redness of joints; grade 3 was ascribed to extensive swelling and redness of joints without evidence of impairment in the mobility of the limb; grade 4 was attributed to a limb with severe joint inflammation associated with impairment of movements of the limb. Values of arthritis severity were obtained for the different experimental groups of rats. These numerical data were statistically compared using Student's *t*-test; two populations were considered to be significantly different if $P < 0.05$ (labelled with an asterisk in Fig. 1).

Immunoblotting (Western blotting)
The method was employed to investigate changes in the repertoire of specificities of antibodies of the different experimental groups of Lewis rats with regard to mycobacterial antigens. Whole cell homogenates of *Myco. bovis*-BCG bacilli were obtained by ultra-sonication of the bacteria for six periods of 30 s in a Branson sonifier set at 100 W [19]. Intact or partially disrupted cells were
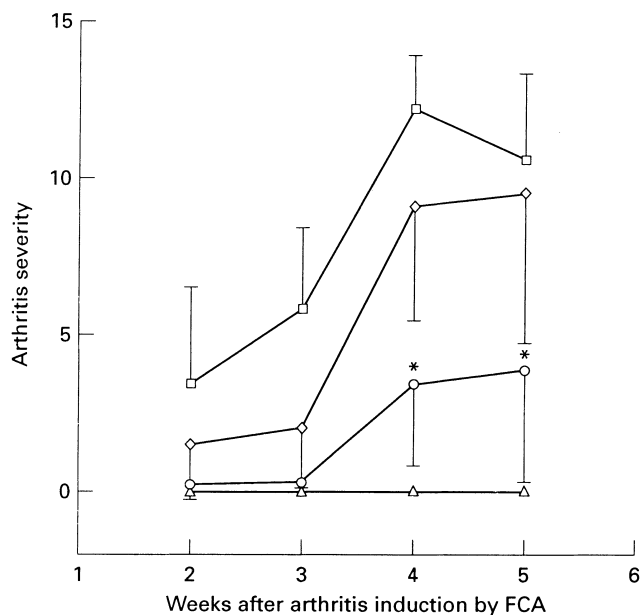

Fig. 1. Comparison of the severity of adjuvant arthritis (AA) among Lewis rats submitted to neonatal injection of either 10^8 viable *Mycobacterium bovis*-BCG bacilli (△), or 50 μg mycobacterial 65-kD heat shock protein (◇), or lactoferrin (LF) (○), or bovine serum albumin (BSA) (□); all animals were also submitted to the arthritogenic Freund's complete adjuvant (FCA) immunization when they were 8 weeks old. The severity of AA is comparable in the groups of rats submitted to neonatal injections of BSA or LF; a decreased severity in AA is seen in animals that received LF neonatally. *Statitistically significant differences with the BSA- and hsp65-treated groups, $P<0.05$. Lewis rats neonatally infected with 10^8 BCG bacilli did not show any signs of joint inflammation after immunization with FCA, thus scoring zero in arthritis severity.

pelleted by centrifugation (6000 *g*) and discarded. A protease inhibitor (phenyl-methyl-sulphonyl fluoride; Sigma) was added to the suspension to a final concentration of 0·1 mM. The supernatants were then used as whole-cell homogenates. Protein extracts of *Myco. bovis*-BCG were concentrated in acetone at –20°C, centrifuged, and dried. They were resuspended and denatured in sample buffer, boiled at 100°C for 2 min. The mycobacterial proteins were separated by SDS–PAGE (7·5% polyacrylamide) at 20 mA for 1–2 h. To determine molecular weights, we used standard low and high molecular weight reference samples (purchased from Sigma); these were complemented with comparisons with coomassie blue-stained SDS polyacrylamide gels of whole-cell mycobacterial proteins and also with published records of major protein bands of mycobacteria [20,21]. The separated proteins were electroblotted overnight onto nitrocellulose membranes [22]. The nitrocellulose protein blots were washed in PBS and blocked for 1 h in PBS containing 5% skimmed milk. They were incubated with 1:1 and 1:20 dilutions in PBS of sera from treated and untreated Lewis rats. The sera were washed out with two changes of PBS, 0·1% NP40, followed by a 10-min wash in PBS. A second incubation was done with sheep anti-rabbit immunoglobulin conjugated with horseradish peroxidase (HRP; Amersham, Aylesbury, UK) in a 1:300 dilution in PBS. The peroxidase reaction was initiated by 2·8 mM of 4-chloro-1-naphtol and 0·015% hydrogen peroxide in PBS, and stopped by washing the blots in water.

RESULTS

Effect of neonatal treatments on incidence and severity of arthritis

We have investigated the effect of different inocula, that were intraperitoneally injected into Lewis rats at the neonatal period, on the incidence and severity of arthritis that was later induced in the same rats when, as adults, they were immunized with FCA. We used different inocula of *Myco. bovis*-BCG bacteria (10^6 and 10^8 viable bacilli), as well as antigens, such as hsp65 and LF, that were previously proposed as having a role in the etiopathogenesis of AA. Our findings are shown in Table 1.

We found that infection of newborn Lewis rats with 10^8 *Myco. bovis* BCG bacilli was the only treatment that changed the classical arthritogenic response observed in adult Lewis rats after immunization with FCA. In fact, all of the Lewis rats that were neonatally infected with 10^8 *Myco. bovis*-BCG bacilli became resistant to FCA-induced arthritis. In control groups of Lewis rats (i.e. rats submitted to neonatal injection of saline, the vehicle of the mycobacterial inoculations), the incidence of FCA-induced arthritis in adult animals was between 80% and 90%; this is comparable to the incidence of arthritis in untreated adult Lewis rats that were submitted to FCA immunization (Table 1).

Interestingly, decrease in the number of bacilli (from 10^8 to 10^6) injected at the neonatal period, or 10^8 BCG infection performed at 3 weeks of age, failed to prevent AA produced by FCA in adult Lewis rats. Neonatal injection of hsp65, LF or BSA had no significant effect on the incidence of AA in adult Lewis rats immunized with FCA. There was, however, a delay of 1–2 weeks in the expression of AA in FCA-immunized Lewis rats that had been submitted to neonatal injection with hsp65 or LF; this delay was not observed in rats that were neonatally injected with BSA.

We also used a quantitative scoring method to compare the

Table 1. Effect of different neonatal treatments of Lewis rats on the incidence of adjuvant arthritis (AA) induced in the same animals (as adults) by immunization with a variant of Freund's complete adjuvant (FCA)

Treatments			Arthritis incidence %
At Birth	Week 3	Week 8	Weeks 10–12
–	–	FCA	83 (10/12)
Saline	–	FCA	83 (10/12)
10^8 BCG	–	FCA	0 (0/10)
	10^8 BCG	FCA	100 (6/6)
10^6 BCG	–	FCA	86 (6/7)
hsp65	–	FCA	86 (6/7)
LF	–	FCA	64 (7/11)
BSA	–	FCA	100 (8/8)

Neonatal infection of the rats with 10^8 *Mycobacterium bovis*-BCG bacilli is the only treatment that protects the animals from AA; the same infection done after the neonatal period does not prevent AA. Neonatal injection of antigens that have been implicated (mycobacterial hsp65 and lactoferrin (LF)) or not (bovine serum albumin (BSA)) in the etiopathogenesis of AA had no significant effect on the incidence of AA triggered by FCA in adult Lewis rats.

severity of arthritis observed in the different groups of neonatally treated Lewis rats (Fig. 1). This evaluation showed that the severity of arthritis in the Lewis rats that were neonatally injected with BSA or hsp65 was not significantly different from that of control rats (i.e. animals submitted to neonatal injection of saline); Lewis rats that had received LF at the neonatal period presented a significant decrease in severity of arthritis at 4–5 weeks after the arthritogenic FCA immunization (Fig. 1).

Repertoire of antibody specificities

Western blotting revealed that sera from Lewis rats submitted to neonatal immunization with *Myco. bovis*-BCG viable bacilli and to FCA immunization (at 8 weeks of age) contained anti-mycobacterial antibodies with different antigenic specificities from control sera (i.e. from rats submitted to neonatal saline injection and FCA immunization at 8 weeks of age).

Lewis rats that developed AA as a consequence of FCA immunization showed anti-mycobacterial circulating antibodies with a wide repertoire of specificities (Fig. 2, lane 1). The major protein bands of mycobacterial antigens that were labelled by these sera were the following: 90 kD, 65 kD, 38 kD and 32 kD. In contrast, Lewis rats that were neonatally infected with 10^8 *Myco. bovis*-BCG bacilli, and thus made resistant to FCA-induced arthritis, presented seric antibodies with a narrower repertoire of antigenic specificities for mycobacterial proteins: these sera failed to recognize the 90-kD and the 32-kD bands and faintly labelled the hsp65 antigen; in contrast, the 19-kD band was more strongly marked by the sera (Fig. 2, lane 2).

Interestingly, Lewis rats submitted to neonatal injection of LF, a procedure that ameliorates but does not prevent FCA-induced arthritis, also showed strong labelling of the 19-kD mycobacterial antigen; sera from these rats also presented labelling of the 32-kD and of the 65-kD band, as was seen in sera from rats with classical FCA-induced arthritis (Fig. 2, lane 3).
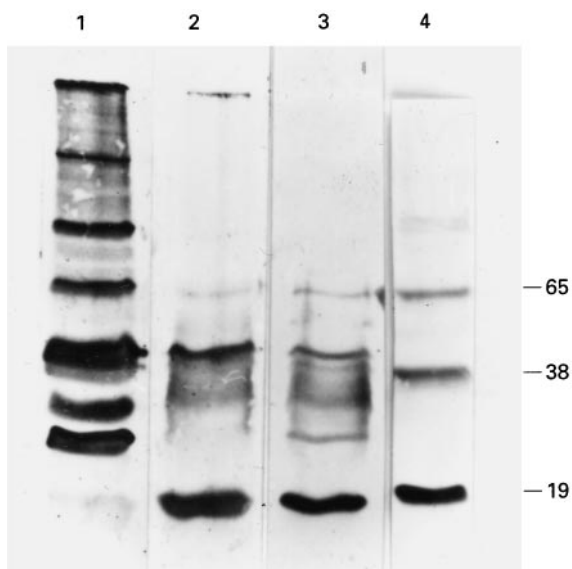
Fig. 2. Comparison by Western blotting of antigen specificities of anti-mycobacterial antibodies present in sera of Lewis rats submitted to different treatments before the induction of adjuvant arthritis (AA) by Freund's complete adjuvant (FCA) immunization. Lane 1, sera of Lewis rats submitted to AA without any other treatment (control); lane 2, sera of rats submitted to neonatal infection with 10^8 *Mycobacterium bovis*-BCG bacilli and to FCA immunization as adults; lane 3, sera of rats submitted to neonatal injection of lactoferrin (LF) and to FCA immunization as adults; lane 4, major proteins of known molecular weights (90 kD, faint band; 65 kD, 38 kD, 19 kD) of *Myco. tuberculosis* used here as standards for molecular weight determination. Neonatal treatment of Lewis rats (infection with 10^8 *Myco. bovis*-BCG bacilli or LF injection) narrows the repertoire of specificities of anti-mycobacterial antibodies present in sera of the animals.

DISCUSSION

Neonatal tolerization of pathogenic autoantigens is an experimental procedure that has been proposed as a potentially useful strategy to prevent autoimmune disease [23–26]. The application of this type of immune intervention requires that the autoimmunity-inducing antigens have been identified. This applies to the Lewis rat model of AA, since the antigens that trigger this autoimmune disorder are known to be present in mycobacteria [3,4,9]. The arthritogenic antigens of mycobacteria induce AA in Lewis rats probably due to molecular mimicry between microbial and mammalian proteins [4,27–29].

We report here that neonatal infection of Lewis rats with mycobacteria results in resistance of the adult animals to the induction of arthritis by FCA immunization. Our finding indicates that animals that are prone to mycobacteria-induced reactive arthritis can overcome their susceptibility to the autoimmune disease if neonatal infection is performed. Our data also show that the neonatally acquired resistance to arthritis is dose-dependent, since it was not observed when the inoculum of mycobacteria was reduced from 10^8 to 10^6 viable bacilli.

We found that the protection from mycobacteria-induced autoimmunity reported here was associated with changes in the repertoire of antibody specificities for mycobacterial antigens; a major change observed in the neonatally treated group was in antibody reactivity against the hsp65 antigen, which was low or

absent in the rats vaccinated against the induction of reactive arthritis. This finding is in agreement with previous data indicating that immune reactivity to hsp65 plays a central role in the etiopathogenesis of AA [3,30,31]. However, anti-hsp65 immunoreactivity may not be sufficient, by itself, to abort AA, since neonatal injection of hsp65 alone did not protect from the autoimmune reaction; this is also consistent with the reported failure of hsp65 alone to be able to induce arthritis in adult Lewis rats [4,9,29].

BCG immunizations have been used in the treatment of adult patients with cancer; it was shown that these immunizations caused reactive arthritis in some of the patients [1,32], resembling that seen in adult Lewis rats immunized with FCA. As we found in Lewis rats, immunization of newborns with mycobacteria may have the opposite effect, i.e. to prevent the expression of reactive arthritis later in life.

In conclusion, we show here that neonatal injection of viable mycobacteria, but not the administration of the hsp65 mycobacterial antigen, is a suitable procedure to make Lewis rats resistant to autoimmune arthritis, and that this alteration of reactivity of the rats is associated with changes in the antibody repertoire of the animals. Further studies are needed to define the mechanisms involved in this phenomenon of acquired resistance to reactive autoimmunity, namely on the amelioration of arthritis observed after neonatal injection of lactoferrin.

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