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Suppression of adjuvant arthritis in rats by intraperitoneal *Mycobacterium butyricum*

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INTRODUCTION

The ability to specifically suppress autoreactive immune response is one of the primary goals in the development of new strategies for the treatment of autoimmune diseases. In order to study antigen-driven peripheral tolerance, we initiated a series of experiments on adjuvant arthritis (AA), an extensively studied model which shares many clinical and histopathologic features with human rheumatoid arthritis. AA is a cell-mediated chronic disease, induced by injection on rat plantar surface of heat-killed mycobacteria suspended in mineral oil (Freund's complete adjuvant) and characterized by chronic joint inflammation. Studies on pathogenesis of adjuvant arthritis suggest an important role for the 65-kD mycobacterial heat shock protein (HSP), both in inducing and in suppressing the disease [1,2]. In previous studies we showed that the i.p. treatment of rats with *Mycobacterium butyricum* on days 3 and 10 after arthritis induction at concentrations 10 times lower than the inducing one, led to a significant, antigen-specific and long-lasting suppression of arthritis development [3]. The role and importance of the Th1/Th2 balance, cytokines and other mediators such as nitric oxide in immunopathological conditions is outlined by several experimental and clinical observations. Recently other regulatory T cell clones have been identified [4].

The main objective of this study was to investigate the role of peritoneal macrophages in preventing arthritis development in rats receiving the antigen specific protection through intra-peritoneal route. We also investigated the possibility of transferring the arthritis protection to naive rats by i.v. injection of lymphonodal or peritoneal cells derived from protected arthritic rats.

MATERIAL AND METHODS

Induction and evaluation of AA

AA was induced in inbred male Lewis rats (Charles River, Italy) weighing 125-150 g, by injecting into the hindpaw 0.6 mg of heat-killed *M. butyricum* (Mb), (Difco, Detroit, MI, USA) suspended in 0.1 ml of paraffin oil (CFA; Complete Freund Adjuvant).

Animals were weighed every 3 days and at the same time the severity of arthritis was assessed by measurement of contralateral paw swelling with an electronic water plethysmometer (Mod. 7150, Ugo Basile, MI, Italy) At days 14, 21 and 28 primary and secondary arthritic lesions were scored on an arbitrary scale (arthritic index) as follows: left and right hindpaws each 0-7, left and right forepaws each 0-4, tail 0-5, ears 0-2, nose and eyes each 0-1.

Treatment

Arthritic animals were randomly assigned to three different treatment groups (20 animals each) as follows:

1. i.p. injection of 0.1 ml of paraffin oil on days 3 and 10 after arthritis induction,
2. i.p. injection of 60 µg of Mb suspended in 0.1 ml of paraffin oil at the same times,
3. i.p. injection of 60 µg of heat-killed *Escherichia coli* in 0.1 paraffin oil at the same times.

A group of 6 healthy rats was also included.

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Peritoneal lavages and macrophages collection

At day 21, 5 rats of each group were killed to obtain peritoneal cells. Cells obtained by peritoneal lavages were washed three times in sterile PBS, counted and suspended at a concentration of $1x10^6$/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with FCS 10%, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.5 mM L-glutamine, 2 mM β-mercaptoethanol, plated in a 24-well microplate and incubated at 37°, 5% CO2. After 2 h, plates were washed to remove non-adherent cells and macrophages were cultured for 24 h. Then culture media were collected and stored at -20°C until use.

Serum IL-6 bioassay

Blood samples were collected by cardiac puncture from three rats of each group at weekly intervals. Blood was centrifuged and the sera were stored at -20°C until testing for IL-6 activity.

To measure IL-6 activity, the murine IL-6 dependent hybridoma cell line 7TD1 was used, according to a previously described method [3]. The standard used in this assay was a human recombinant IL-6 (Boehringer, Mannheim, Germany).

Plasmatic nitrite/nitrate and peritoneal macrophage nitrite measurement

Plasma nitrite/nitrate levels were assayed using a nitrite/nitrate assay kit (Cayman, Ann Arbour, MI, USA). Briefly, in a 96-well microplate 40 µl of plasma, 40 µl of assay buffer, 10 µl of enzyme co-factor and 10 µl of nitrate reductase were placed. Samples were incubated for 3 h at room temperature to allow a complete conversion of nitrate to nitrite. To evaluate the total nitrite amount, 100 µl of Griess reagent (1:1 of 0.1% naphthylethylenediamine dihydrochloride / 1% sulfanylamide in 5% H_3PO_4) were added, allowing the color to develop for 10 min and reading the absorbance at 540 nm using a microplate reader. Concentration of total (nitrite + nitrate) plasmatic NO was determined using a 0-35 µM range nitrate standard curve.

Macrophage culture media were assayed for nitrite production using Griess reagent. For this purpose, 100 µl of Griess reagent were added to 100 µl of sample allowing the color to develop for 10 min and reading the absorbance at 540 nm using a microplate reader. Nitrite concentration was determined using a 0-35 µM range nitrite standard curve.

Flow cytometry

Cells obtained by peritoneal lavages after washing, were incubated for 15 min at 4°C with mouse anti-rat ED1 antigen (FITC) expressed by the majority of tissue macrophages (CD68-like) and with mouse anti-I-A Class II Monomorphic antigen (PE) (SEROTEC Ltd, Oxford, England), which increases on macrophage surface with the activation. Cells were washed twice with PBS and fixed with 2% paraformaldehyde. The analyses were performed for 10,000 events in each sample by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Adoptive transfer

Peritoneal cells and lymphocytes for adoptive transfer were driven from donor-protected and unprotected arthritic rats 14 days after arthritogenic treatment. Animals were killed by bleeding and the peritoneum was washed with cold PBS. Cells were washed twice, suspended in complete medium at a concentration of 3×10^7/ml. From the same rats, poplyteal and mesenteric lymphonodes were removed, crumbled, filtered, and suspended in complete medium at a concentration of 3×10^7/ml. Recipient naive rats were injected i.v. with 1 ml of peritoneal or lymphonodal cells.

RESULTS

The i.p. administration to rats of low doses of specific antigen (Mb), 3 and 10 days after arthritis induction, significantly inhibited disease development, documented by the reduction of contralateral hindpaw volume (from 2.28 ml 0.39 in untreated rats to 1.63 ml 0.38 in Mb i.p. treated rats on the 21st day) and arthritic index (from 24.9 ± 3.15 in untreated rats to 14.45 ± 4.82 in Mb i.p. treated rats on the 21st day) which remained significant until the 28th day. The injection of rats with the uncor-

related antigen *E. coli* at the same conditions did not reduce the severity of arthritis.

On the 21st day after arthritis induction, which coincides with the maximum expression of disease, a rise in plasmatic levels of IL-6 and NO_2^-/NO_3^- were detected in arthritic rats compared to healthy animals. In arthritic rats treated with Mb a significant further rise in NO_2^-/NO_3^- levels and a significant decrease in IL-6 levels were observed, while the group treated with *E. coli* did not show significant differences when compared to the arthritic one (*Table 1*). Production of NO_2^- and IL-6 by macrophages collected from peritoneal cavity on day 21 appeared to be higher in the three groups of arthritic animals in comparison to healthy but no differences were observed in relation to treatments. Macrophages from Mb-treated rats showed a significantly higher expression of the activation marker I-A Class II Monomorphic antigen assayed by FACS ($p< 0.05$ compared to arthritic) (*Table 1*).

To determine whether the protection against arthritis can be transferred, lymphonodal and peritoneal cells were prepared from antigen-protected and unprotected rats on day 14, and adoptively transferred into naive animals, followed, 15 h later, by subplantar Mb immunization. As shown in *Figure 1*, recipients of peritoneal cells from Mb-protected rats showed a significant reduction in clinical severity of AA, expressed as arthritic index. However no significant suppression of AA was observed when lymphonodal cells from protected rats and lymphonodal or peritoneal cells from unprotected rats were transferred.

DISCUSSION

AA, an extensively studied experimental model that shares many features with rheumatoid arthritis, can be prevented or treated via administration of the causative antigen or of

TABLE 1 - *Comparison among different in vitro parameters on day 21 of adjuvant arthritis (AA) in plasma and in macrophage culture medium (see Materials and Methods). Data represent the mean ± SD. ** $p < 0.01$, * $p < 0.05$ significantly different from AA group (Student's t-test).*

	PLASMA		MACROPHAGES		
	NO_2^-/NO_3^- (μM)	IL-6 (U/ml)	NO_2^- (μM/10^6 cells)	IL-6 (U/ml)	I-A class II (MFI§)
HEALTHY	60.85 ± 14.14	3.00 ± 0.97	5.29 ± 4.74	27.10 ± 11.70	14.25 ± 3.59
AA	100.80 ± 17.16	73.12 ± 31.80	71.47 ± 14.90	121.81 ± 66.90	101.75 ± 16.34
AA+Mb	187.72 ± 63.13 *	22.49 ± 4.81 **	74.05 ± 21.76	118.80 ± 87.90	130.60 ± 25.36 *
AA+Ec	105.49 ± 28.17	55.75 ± 18.36	59.56 ± 15.65	87.75 ± 41.28	75.25 ± 19.97

§ Mean fluorescence intensity

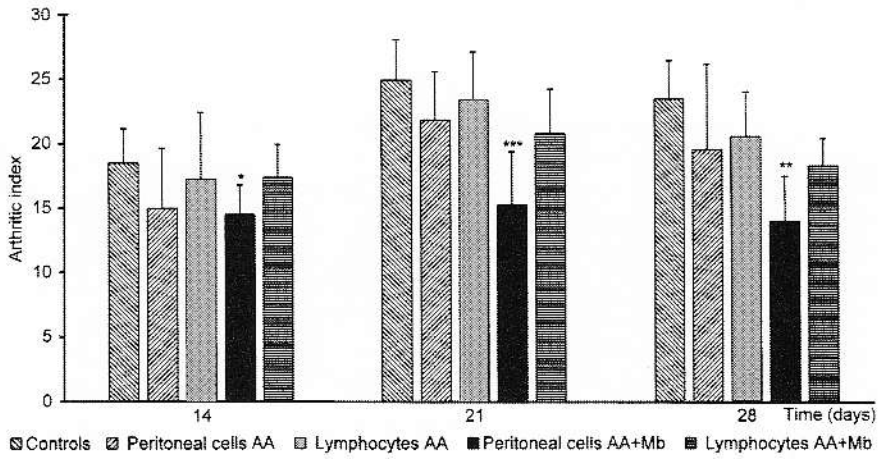

FIGURE 1 - Arthritic index (range 0-32) of adjuvant arthritis in rats receiving i.v. 1 ml of medium (controls), $30x10^6$ peritoneal or lymphonodal cells/ml from arthritic animals (peritoneal cell AA and lymphocyte AA), or $30x10^6$ peritoneal or lymphonodal cells/ml from protected animals (peritoneal cell AA + Mb and lymphocyte AA + Mb).
Data represent the mean ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ significantly different from control group (Mann-Whitney's U test).

the correlated heat shock proteins also given by oral route. Our data show that the i.p. injection of *M. butyricum* was effective in inhibiting arthritis development, when given 3 and 10 days after the arthritogenic inoculum. Several mechanisms of peripheral tolerance have been proposed and documented *in vivo* and *in vitro* and their understanding can be clinically relevant for the treatment of human autoimmune diseases [6]. In our model the strong decrease in IL-6 plasma levels observed in protected animals is consistent with an immune deviation, where a functional shift from a CD4+ T helper 1 (Th1) to Th2 response occurs. The increase of nitrite/nitrate levels in plasma of protected animals is a unexpected finding. A possible regulatory role of nitric oxide in the arthritic process remains to be investigated.

It is known that many of the alterations in lymphocyte function are mediated, at least in part, by activated macrophages. Moreover, among the factors that preferentially promote immune deviation, the role of antigen-presenting cells such as macrophages should be taken into account. These considerations, together with the fact that the protection was obtained using the i.p. route of administration, led us to investigate some functional properties of peritoneal macrophages. On day 21 after arthritis induction, peritoneal macrophages from antigen-treated rats did not differ from those collected from untreated rats, as far as production of nitric oxide and IL-6 are considered, indicating that they are not functionally suppressed. Moreover the higher expression of I-A Class II Monomorphic antigen is an index of cell activation, suggesting that Th2 or other suppressive cells could be activated via macrophages.

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