Immunology 1994 **81** 414–419

Cell migration studies in the adoptive transfer of adjuvant arthritis in the Lewis rat

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SUMMARY

Adjuvant arthritis (AA) can be induced in Lewis rats by a single injection of either heat-killed *Mycobacterium tuberculosis* or the lipoidal amine CP20961. Concanavalin A (Con A)-stimulated T cells isolated from AA rats are able to adoptively transfer the disease to naive syngeneic recipients. It is unclear, however, whether these transferred cells traffic directly to the joint and initiate arthritis, or whether secondary host cells are responsible for activation of the disease. In the current investigation, T cells labelled with the vital fluorescent dyes Hoechst H33342 and Zynaxis PKH26-G were used to adoptively transfer adjuvant disease to naive recipients. At various stages of disease development sections of ankle joints, together with a range of soft tissues, were examined by fluorescence microscopy to determine the distribution of labelled donor cells in the recipients. Intensely fluorescent lymphocytes were observed in the liver, spleen and lymph nodes within 24 hr of adoptive transfer. Foci of such cells were clearly visible in the primary lymphoid tissues as late as 14 days after transfer. However, close examination of both ankle joint sections and patellar fat pad cells throughout the time–course of the study failed to detect any labelled cells at the lesion site. To develop these observations further, we performed adoptive transfers to nude Lewis rats (rnu/rnu) and found that they were only moderately sensitive and developed, at best, a transient arthritis. This observed difference could not be explained by a generalized lack of an inflammatory response, since we were able to elicit a zymosan peritonitis in the nude rats. However, in nude Lewis rats a striking increase in adoptively transferred AA was obtained after reconstitution with 4×10^8 naive syngeneic spleen cells. These combined observations suggest that a host-derived immune cell population is crucial for arthritis induction in the adoptive transfer system.

INTRODUCTION

Rat adjuvant arthritis (AA) is a well-defined model of human rheumatoid arthritis that can be induced by immunization of Lewis rats either with *Mycobacterium tuberculosis* (Mtb) suspended in Freund's incomplete adjuvant (FIA),[1] or with the lipoidal amine CP20961 in paraffin oil.[2] Fourteen days after induction, a progressive infiltrate of mononuclear cells is seen in the ankle joints, with concomitant early signs of bone remodelling. Several studies have already shown the important role of T lymphocytes in this arthritis model: AA cannot be induced in athymic (nude) rats;[3] treatment of rats with anti-T-lymphocyte antibodies prevents the induction of disease;[4,5] and, finally, the disease can be transferred to naive or irradiated syngeneic recipients with concanavalin A (Con A)-expanded T-cell lines[6] or clones.[7]

The possibility that T lymphocytes can migrate specifically to the inflammatory site has been examined in several models recently. In a dermal inflammation model, it has been demonstrated that a unique, small population of peritoneal exudate lymphocytes can migrate preferentially to cutaneous inflammatory sites[8–11] and reside there for long periods of time. This contrasts with the kinetics of lymphocyte migration into the arthritic joint, which appears to be a major traffic site for all lymphocytes, although they appear to reside there for only a relatively short time.[12] Furthermore, reports on the autoimmune disease experimental allergic encephalomyelitis (EAE), show that only a small percentage of transferred myelin basic protein (MBP)-reactive T cells accumulates in the central nervous system[13,14] and that non MBP-reactive lymphocytes ('bystander cells') can also play a role in mediating passively transferred EAE.[15]

Recently, DeJoy *et al.*[16] reported that in the adoptive transfer of AA some of the donor T cells (adjuvant sensitized) migrate to the synovium, where they may be responsible for disease induction. However, subarthritogenic doses of affinity-purified anti-collagen IgG were co-injected to facilitate adoptive transfer of AA, thereby inducing a more rapid onset of disease. Since this lesion appears to be complement dependent,[17] the co-injected anti-collagen IgG may stimulate the influx of inflammatory cells, including the transferred AA cells. Thus it still

Received 16 August 1993; revised 6 October 1993; accepted 4 November 1993.

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remains to be demonstrated in the 'classical' adoptive transfer system,[6] which is primarily mediated by cellular immune mechanisms, whether donor T lymphocytes are capable of specific migration to joints and subsequent initiation of arthritis.

We therefore carried out adoptive transfer experiments of AA to naive recipients using either H33342-[18] or PKH26-[19] labelled, Con A-stimulated T cells. At various stages of disease development, ankle joints and a range of soft tissues were examined by fluorescence microscopy to investigate whether any donor T lymphocytes could be detected. Additionally, adoptive transfer experiments were performed on immunocompromised recipients to determine whether a host-derived cell population plays an important role in the induction of disease in the adoptive transfer system.

We were not able to detect any labelled cells in the joints, suggesting that the cellular infiltrate at the lesion site is host derived. Transfer experiments to nude Lewis recipients supported this hypothesis, since only moderate arthritis developed in these animals.

MATERIALS AND METHODS

Rats

Female Lewis rats were originally obtained from the Zentral Institute fur Versuchstierzucht (Hannover, Germany) and bred in our own facilities, or were from Olac, Bicester, U.K. Female Lewis rnu/rnu were obtained from Mollegaard (Skensved, Denmark). The rats weighed between 140 and 170 g at the beginning of each experiment and were fed standard food and tap water *ad libitum*.

Adoptive transfer of adjuvant arthritis

The technique used was that described by Taurog *et al.*[20] Briefly, female inbred Lewis rats were injected intradermally at the base of the tail and the hind paws with 250 μg of Mtb (heat-killed human strains C, DT and PN, Central Veterinary Laboratory, Weybridge, U.K.). The Mtb was finely ground in a pestle and mortar and suspended evenly in light paraffin oil at a concentration of 2·5 mg/ml. In case of the PKH26-GL labelling experiments, donor cells originated from CP20961 (Pfizer, Groton, CT) (5 mg/rat in 0·1 ml paraffin oil)-primed animals. Ten days after injection, popliteal, inguinal and subaortic lymph nodes and spleens were removed aseptically. After a single-cell suspension was made, the T lymphocytes were purified by passage over a nylon-wool column. T cells were then stimulated with Con A (Flow Labs, McLean, VA) (3 μg/ml) for 48 hr in RPMI supplemented with 10% fetal calf serum (FCS), 10 mM pyruvate, 20 mM glutamine and 40 μg/ml gentamycin (Flow Labs) at a concentration of $2{\cdot}5 \times 10^6$ cells/ml. After incubation, T lymphocytes were washed in phosphate-buffered saline (PBS), and either labelled or immediately injected intravenously into naive syngeneic recipients or irradiated recipients, as stated (5×10^8 cells/recipient). Arthritis developed within 5 days and was scored macroscopically by measuring hind paw thickness with an industrial micrometer, or using the scoring system of Billingham *et al.*[21]

Labelling of transferred cells

At the end of the *in vitro* Con A stimulation, AA T cells were harvested and washed three times with PBS at room temperature. These viable cells were then stained with either H33342 (Calbiochem, La Jolla, CA) or PKH26-GL (Zynaxis, Malvern, PA) dye. The H33342 labelling was performed according to methods of Loeffler *et al.*[18] Briefly, lymphocytes were resuspended in a serum-free Hanks' balanced salt solution (HBSS) containing 1 μM H33342 at a concentration of 10^6 cells/ml, and incubated for 30 min in a 37° water bath with gentle agitation. Cells were then washed twice in HBSS and viability and fluorescence were assessed by microscopic observation. Labelled T cells were then used to transfer arthritis to naive animals.

The PKH26-GL labelling was performed according to methods of Horan *et al.*[19] Briefly, 1×10^8 cells suspended in 5 ml of the kit (Zynaxis) diluent was added to a further 5 ml of diluent containing 4 μM PKH26-GL and incubated for 2–3 min at room temperature with gentle shaking. The reaction was stopped by the addition of an equal volume of FCS for 5 min. Twenty-five millilitres of RPMI supplemented with 10% FCS was subsequently added and the cells were pelleted by centrifugation at 400 *g* for 10 min. Cells were then washed twice, once with RPMI supplemented with 10% FCS and once with PBS. The fluorescence and viability were assessed by microscopy before intravenous (i.v.) injection into naive recipients.

In vitro *mitogen stimulation*

In order to assess the effects of cell division on fluorescence intensity, labelled donor T lymphocytes were maintained *in vitro* in the presence of Con A (3 μg/ml) for 7 days. The cultures were examined daily and the percentage labelling established by determining the ratio of fluorescent to non-fluorescent cells. This was accomplished by counting the same haemocytometer field under both fluorescent and normal illumination.

In vivo *cell traffic experiments*

Adoptive transfer experiments with H33342-labelled cells were performed four times and these data comprise the following days post-transfer: 1, 2, 3, 4, 5, 7, 9, 10, 13 and 14. Adoptive transfer experiments with PKH26-GL-labelled cells were performed twice and comprise days 2, 5, 7, 9 and 13 post-transfer. At all the above time-points, groups of three recipients were killed; and inguinal and popliteal lymph nodes, thymus, spleen, liver and ankle joints were removed and immediately submerged in liquid nitrogen. Multiple cryostat sections were cut and examined by fluorescence microscopy for the presence of labelled cells.

In the lymph nodes, at least three serial sections at different levels in the block were examined. For the larger organs, e.g. liver and spleen, at least three sections were taken, again at multiple levels in order to obtain as representative a sample as possible. Three sections from at least two levels were examined in the joints. Peripheral blood samples were collected from the dorsal aorta, and leucocytes isolated by sedimentation in 0·5% methyl cellulose. The percentage of labelled cells in the buffy coat was then determined as described above.

Patellar fat pad cells

At days 2, 5, 7 and 9, two recipients were killed and the patellar fat pads removed. Single-cell suspensions were made by enzymatic digestion using the method described by Dejoy *et al.*[17] Briefly, the patellar fat pads were digested in serum-free RPMI supplemented with 0·5 mg/ml collagenase, 0·15 mg/ml DNase and 0·05 mg/ml hyaluronidase (Sigma Chemical Co., St Louis, MO). After 90 min of gentle agitation at 37°, the cell suspension

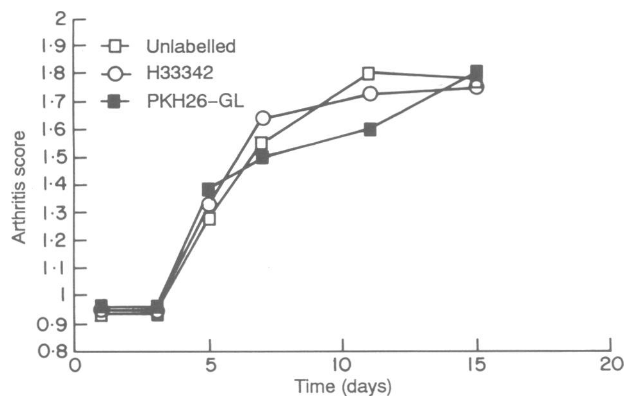

Figure 1. Comparison of the severity of AA after adoptive transfer with either labelled (H33342 or PKH26-GL) or non-labelled T lymphocytes. Arthritis was scored as the sum of paw thickness of both hindlegs (cm). Each group consisted of four animals.

was centrifuged at 400 ***g*** and the cells washed with RPMI. This suspension was filtered through a 60-μM Nytex filter (Tetko, Elmsford, NY), and at least 100 cells examined by fluorescence microscopy.

Irradiation of the recipients

Groups of four recipient Lewis rats were exposed to 2·5 or 5 Gy total body irradiation (TBI). The physical parameters of the X-irradiation were: 18 MV; field size, 40×40 cm^2; dose rate 2 Gy/min. One hour after irradiation, arthritis was induced by adoptive transfer of Mtb-primed, non-fluorochrome-labelled T cells.

Induction of sterile peritonitis

Four rats from the control, non-reconstituted nude and 5 Gy-irradiated groups were injected intraperitoneally (i.p.) with 18 ml of saline containing 1% glycogen (Merck, Darmstadt, Germany) or 5 mg of zymosan (Sigma Chemical Co.). Irradiation was performed either 2 or 5 days before induction of the peritonitis. Sixteen hours after injection, rats were killed and the peritoneal cavity washed once with 40 ml of saline. The elicited peritoneal cells were washed; the total cell number determined using a Coulter counter; and differential leucocyte counts performed on Wright–Giemsa-stained cytospin preparations. At least 200 nucleated cells were identified as polymorphonuclear cells (PMN) or monocytes (Mo).

RESULTS

In vitro mitogen stimulation

H33342-labelled lymphocytes were cultured *in vitro* with Con A to investigate whether, during proliferation, the dye was lost due to dilution. Initially, all cells took up the label and, following 7 days of *in vitro* stimulation with Con A, 92% of viable cells remained fluorescent. PKH26-GL has already been shown to be detectable *in vivo* after 121 days.[22,23] Previous transfer experiments had shown that recipient animals developed macroscopic and histological signs of arthritis as early as 4 days following injection of donor cells. These data therefore suggest that both dyes can be used effectively to determine the distribution of transferred cells during the period of lesion development.

In vivo cell traffic experiments

Since the labelled cells were still clearly fluorescent following proliferation, adoptive transfer experiments were performed with both dyes to ascertain whether either label affected the severity of the arthritis. Labelled AA cells were transferred to naive recipients, where it was found that neither label had any apparent affect on the incidence or severity of the arthritis in comparison with non-labelled cells (Fig. 1). Time–course experiments were performed subsequently using either the H33342 or the PKH26-GL dye, which label the cell nucleus and cytoplasmic membrane, respectively. The results of the cell trafficking experiments were identical for both dyes and representative pictures of both dye are shown in Fig. 2.

At day 1 post-transfer, the majority of fluorescent cells were localized in the liver and spleen. Small foci of positive lymphocytes were observed in the popliteal and inguinal lymph nodes. Approximately 5% of the peripheral white cells were stained at this time; this increased to a maximum of 90% on day 2 before falling to non-detectable levels at all subsequent time-points. At days 2 and 3, the number of clearly labelled cells in the liver and spleen was seen to decrease, whilst their frequency in the lymph nodes increased. From days 4 to 14, foci of clearly labelled lymphocytes were still observed in the lymph nodes and spleen, but at the later time-points their numbers were reduced. During the same period, the liver and spleen developed a progressive autofluorescent appearance which was particularly marked in the animals which received H33342-labelled cells. Macrophages sequestered the dyes and exhibited intense, particulate cytoplasmic fluorescence. Throughout the entire time–course of the experiment, no labelled cells were observed in the thymus.

The examination of ankle joints at all time-points failed to reveal the presence of labelled cells at the lesion site, despite multiple sectioning at different levels of the block. The absence of labelled cells in the joint was not due a lack of lesion development, since in each adoptive transfer experiment macroscopic symptoms of arthritis were clearly observed.

Patellar fat pad studies

Single-cell suspensions of patellar fat pads, prepared by enzymatic digestion, were examined by fluorescence microscopy. No labelled cells could be detected at any time-point.

Adoptive transfer to immunocompromised recipients

To determine whether a host-derived T-cell population is essential for arthritis induction, adoptive transfer experiments to immunocompromised (T-cell depleted) recipients were performed. Rats that had received 2·5 Gy TBI developed a slightly less severe arthritis compared with controls. However, recipients that had received 5 Gy TBI developed a significantly milder arthritis ($P < 0{\cdot}02$; Wilcoxon rank test) (Fig. 3).

Adoptive transfer experiments were also performed with nude Lewis (rnu/rnu) rats, which are profoundly deficient in mature T lymphocytes.[23] These experiments resulted in the initial development of a mild arthritis, which failed to progress ($P < 0{\cdot}01$; Wilcoxon rank test) compared with the control Lewis rats (Fig. 4).

Subsequently, AA was transferred to nude recipients which had been reconstituted with 4×10^8 naive syngeneic spleen cells 1 day before the adoptive transfer. Although no significant

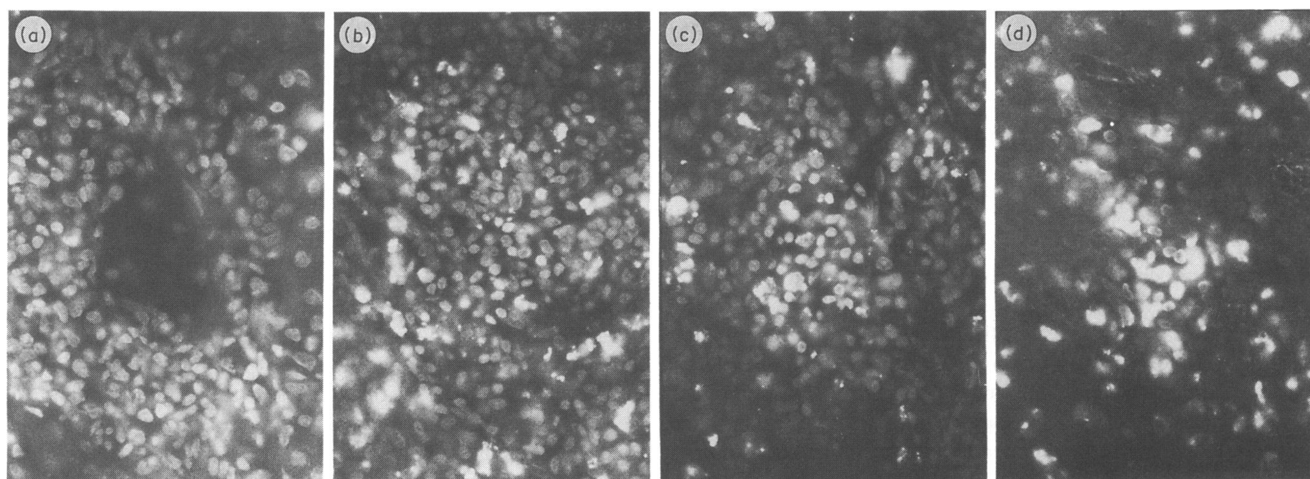

Figure 2. Analysis of the different tissues by fluorescence microscopy. (a) Liver, day 1 after transfer; (b) spleen, day 2 after transfer; (c) spleen, day 4 after transfer; (d) lymph node, day 14 after transfer. Magnification ×240.

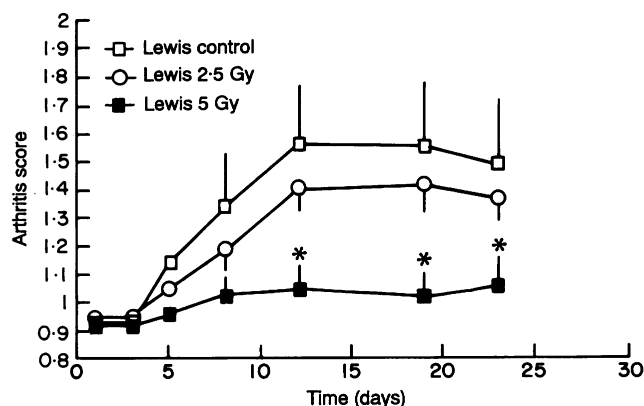

Figure 3. Adoptive transfer of AA to irradiated and non-irradiated recipients. Arthritis was scored as the sum of paw thickness of both hindlegs (cm). Each group consisted of four animals. Data were tested with the Wilcoxon rank test (*$P<0·02$).

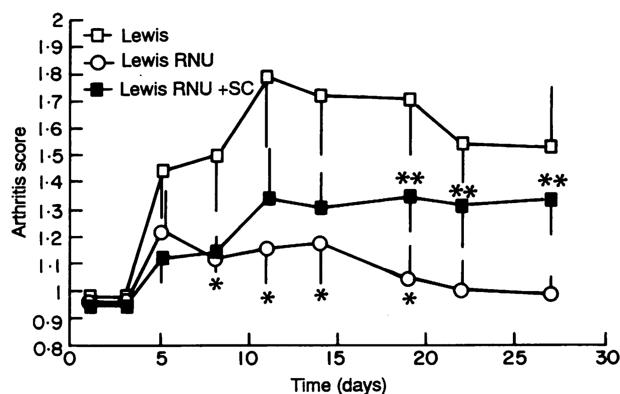

Figure 4. Adoptive transfer of AA to naive Lewis rats, nude Lewis (RNU) rats and nude Lewis recipients reconstituted with 4×10^8 naive spleen cells (SC) 1 day before transfer. Arthritis was measured using a calliper and the values represent the sum of the thickness of both hindpaws (cm). Each point represents 7–14 animals. Data were tested with the Wilcoxon rank test. Differences between the nudes and the naive Lewis rats were significant, *$P<0·01$. Furthermore, the differences between the nudes and the reconstituted nudes were significant, **$P<0·05$.

difference was observed at the onset of disease (nudes versus reconstituted nudes), it appeared that at later time-points the arthritis was significantly more severe and chronic ($P<0·05$; Wilcoxon rank test) in the reconstituted nude recipients (Fig. 4).

It was noted that about 75% of the nude recipients, but not the reconstituted nude rats, died between day 22 and day 30 post-transfer. Macroscopic analysis did not show any signs of graft-versus-host disease.

Induction of sterile peritonitis

Adoptive transfer to T-cell depleted recipients resulted in a mild, non-progressive arthritis. A sterile peritonitis was therefore induced in control, irradiated and nude Lewis rats to determine whether such animals displayed a generalized inability to mount an immune response. In irradiated Lewis rats, it was found that the influx of inflammatory cells was markedly reduced compared to that of control rats. In contrast, the nude Lewis rats

Table 1. Peritoneal recruitment assay in immunocompromised animals

Recipient	*n*	Total cell no. $\times10^7$ obtained from peritoneum	Identification of cells	
			PMN	Mo
Exp. 1				
Lewis	4	4·1±2·3		
Lewis 5 Gy (day −2)	4	0·8±0·2		
Lewis 5 Gy (day −5)	4	0·6±0·4		
Exp. 2				
Lewis	4	8·4±1·0	72±2	28±2
Lewis RNU	4	11·4±0·7	60±4	40±4

Peritonitis was induced in Exp. 1 by injection of 1% glycogen and in Exp. 2 by 5 mg of zymosan.

showed the greatest response, recruiting more inflammatory cells to the peritoneum than any other group (Table 1).

DISCUSSION

Previous studies in rat AA have demonstrated the central role of T lymphocytes in this model.[3-7] Furthermore, it has been reported[17] that a small number of T lymphocytes can be detected by immunocytochemical analysis of the knee synovium from rats undergoing adoptive transfer. Whether the transferred T lymphocytes can migrate specifically to the joint remains a matter of debate. Recently, DeJoy *et al.*[16] have used an adoptive transfer system in which they were able to recover labelled cells from the knee synovium of recipients. However, their system appeared to be complement dependent as anti collagen IgG was co-injected. It thus remains to be demonstrated whether donor cells can be detected in the joint lesion when the disease is transferred by AA-specific lymphocytes in the absence of co-injected IgG. In the current study, cell traffic experiments were undertaken to address this issue.

Confirming earlier studies,[18,22,23] our data indicate that both dyes can be effectively used for *in vivo* cell trafficking experiments without affecting the incidence or severity of arthritis. This suggests that the labelling does not alter the functional characteristics of these cells. Perhaps surprisingly, we were unable to detect any labelled cells in the ankle joints or patellar fat pads at any time-point in our adoptively transferred arthritis; this suggests that the cellular infiltrate within the joint lesion site is predominantly host derived. It has been reported,[16] that T cells are present within inflamed synovium of adoptively transferred AA, and we have seen T cells in the later stages of adoptively transferred disease (unpublished observations) when lesions are well developed. However, the appearance of T cells within lesion sites in the direct form of CP20961, in these Lewis rats, occurs much later than inflammatory macrophages, which enter the synovium and initiate the arthritis several days before T cells arrive (S. C. R. Meacock and D. R. Brandon, manuscript submitted for publication). Our present data, therefore, suggest that the transferred T cells confer arthritogenicity to host cells. This does not totally rule out the possibility that a few transferred T cells, undetected during our studies, entered putative lesion sites and set the stage for arthritis, they were certainly present in lymph nodes at day 14, but the overall lack of appearance of labelled cells at lesion sites infers initiation of the actual arthritis by host cells.

The above findings are in agreement with the unpublished experiments of J. D. Taurog and colleagues (personal communication), who were also unable to detect any ^{111}In-labelled adoptively transferred cells in the arthritic joint. This contrasts with adoptive transfer of EAE, where several investigators have detected small amounts of labelled cells at the lesion site.[13,14] The difference between adoptively transferred EAE and our findings in AA may reflect the nature of the priming antigen. In EAE the donor cells are primed against MBP, an antigen that is present in large amounts at the lesion site. However, it remains unknown which antigens are recognized by Mtb- or CP20961-primed T cells in joint lesions in either direct or adoptively transferred arthritis.

To support our findings further, adoptive transfer experiments were performed on T-cell depleted recipients. Our data show that irradiation of recipients reduced their susceptibility to development of adoptively transferred AA in a dose-dependent manner. This finding can be explained by a general immune dysfunction in these animals, as exemplified by their inability to recruit inflammatory cells to a non-specific peritoneal stimulus. These observations concur with those of Taurog *et al.*,[20] who have demonstrated that bone marrow reconstitution is essential in T-cell depleted recipients for initiation of arthritis.

Adoptive transfer experiments were also conducted in nude (rnu/rnu) Lewis rats, which are reported to lack almost all mature T lymphocytes.[24] The adjuvant disease transferred to these recipients was mild and non-progressive. The lack of disease development could not be explained by a generalized immunodeficiency, since they were able to recruit inflammatory cells in response to an induced peritonitis. Indeed, these nude rats were able to recruit even more macrophages and granulocytes to the peritoneum in comparison with normal Lewis rats. This is perhaps not surprising, since it has been reported previously that nude rats do have higher numbers of circulating monocytes and granulocytes in peripheral blood.[25] Small numbers of mature lymphocytes have been reported in nude rats;[26] this may explain the observation that these animals developed a mild, adoptively transferred arthritis. Other potential mechanisms must not, however, be ruled out, and the role of the macrophage in adoptively transferred AA, for example, remains in question. The high mortality incidence of the nudes at later time-points could be explained by the housing, since they were not maintained under specific pathogen-free conditions.

Interestingly, the data obtained from experimental transfer of AA to nude rats contrast with adoptive transfer of EAE, since in this model the nude rats were as susceptible as the control recipients.[27] Nevertheless, the latter data supports our present finding, that a host-derived T-cell population is essential for the development of arthritis by adoptive transfer. Supporting evidence for this hypothesis was obtained by reconstituting the nude recipients with 4×10^8 naive syngeneic spleen cells. These rats proceeded to develop a more severe arthritis than the non-reconstituted controls.

The cellular interactions responsible for the arthritogenic effect of donor T lymphocytes are difficult to understand. One plausible explanation may be that the passively transferred lymphocytes induce anti-idiotypic T cells in the host lymphoid tissue which are able to migrate specifically to the synovium. These cells have been reported in vaccination experiments in EAE,[28] where anti-idiotypic cells were detected in the popliteal lymph nodes of rats that had been immunized with MBP-primed lymphocytes. An alternative explanation is that transferred T cells may induce the activation of host macrophages which then migrate to the joint and initiate lesion development. The recent observation that AA can be transferred by a macrophage-type of synoviocyte supports this hypothesis.[29] Macrophage involvement would also explain why nude recipients were seen to develop a mild adoptively transferred AA in our experiments. Further experiments are clearly required to test these hypotheses.

In conclusion, our experimental data indicate that in the 'classical' adoptive transfer, which is primarily mediated by cellular immune mechanisms, a host-derived T lymphocyte population is crucial for arthritis development. This is probably controlled within draining lymph nodes where the donor T lymphocytes initiate the host response.

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