Clin. exp. Immunol. (1978) 32, 86–96.

Suppression of rat adjuvant disease by cyclophosphamide pretreatment: evidence for an antibody mediated component in the pathogenesis of the disease

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(*Received* 28 *November* 1977)

SUMMARY

Cyclophosphamide (Cy), given intraperitoneally at a dose of 100 mg per kg body weight 3 days before adjuvant, was found to abolish the development of adjuvant disease in the PVG/c rat. This treatment, however, enhanced the delayed hypersensitivity responses to purified protein derivative of tuberculin (PPD) developed by these animals. Lower doses of Cy caused a partial inhibition of arthritis which was dose-related. When the time between giving Cy and the injection of adjuvant was increased, a gradual time-dependent recovery of the response was observed.

The arthritic response was restored by the passive transfer of $7{\cdot}6 \times 10^7$ to $1{\cdot}5 \times 10^8$ normal syngeneic spleen cells, although the development of secondary lesions was delayed by 7–14 days. The response could also be restored by the transfer of small amounts of serum from arthritic, but not normal, rats. Large amounts of serum failed to restore the response.

Additional evidence that pretreatment with Cy preferentially depleted the B lymphocytes was obtained by the histological examination of the lymphoid tissue. It was also shown that the primary antibody response to sheep erythrocytes was abolished by Cy, but that skin allograft rejection was unaffected.

A partial inhibition of the acute inflammatory reaction to carrageenan was observed 3 days after giving Cy.

It is suggested that the pathogenesis of adjuvant arthritis involves an immune complex-mediated phase, which initiates the joint lesions. Once these lesions have formed, cell-mediated immune mechanisms predominate in the development of the disease. It is not known whether the persistence of immune complexes is necessary to maintain the lesions.

INTRODUCTION

The pathogenesis of adjuvant disease in the rat is thought to result from a cell-mediated immune response to disseminated mycobacterial antigen. This conclusion is based upon the histological findings in the disease (Pearson & Wood, 1959; Burstein & Waksman, 1964; Glen & Gray, 1965), the ability to transfer the disease with lymphoid cells (Waksman & Wennersten, 1963; Whitehouse, Whitehouse & Pearson, 1969; Quagliata & Phillips-Quagliata, 1972), but not serum (Waksman, Pearson & Sharp, 1960), and the ability to inhibit its development with immunosuppressive drugs (Ward *et al.* 1964) and anti-lymphocyte globulin (Currey & Ziff, 1965). It has also been shown by Whitehouse & Whitehouse (1968) that thoracic duct drainage of rats for 24 hr severely depresses the development of adjuvant disease.

Several observations suggest that cell-mediated mechanisms may not entirely account for the develop-

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0099-9104/78/0400-0086$02.00

ment of the disease. Isakovic & Waksman (1965) found that adjuvant disease developed in neonatally thymectomized rats, although other cell-mediated functions were depressed. More recently, Lennon & Byrd (1973) reported that 50% of neonatally thymectomized rats developed a polyarthritis when injected with basic protein of myelin in Freund's complete adjuvant (FCA). In intact rats the addition of even relatively simple proteins, such as bovine serum albumin, to the adjuvant prevents the development of arthritis (Isakovic & Waksman, 1965, Pearson & Wood, 1969).

Hollingsworth, Greenberg & Dawson (1976) showed that rats of the highly susceptible Long Evans strain were still able to develop adjuvant arthritis, albeit less severe, when peripheral blood T lymphocytes were reduced to 20–30% of normal.

Adult thymectomy has also been shown to increase the incidence and severity of arthritis in two low-responder strains of rat (Kayashima, Koga & Onoue, 1976).

Kourounakis, Nelson & Kupusta (1973) found that in rats treated with cobra venom factor the development of adjuvant disease was delayed, suggesting a role for antibody. Perper, Oronsky & Blancuzzi (1972) had previously shown that anti-mycobacterial antibody appeared as early as 4 days after injection and persisted at a fairly high level. Delayed hypersensitivity reactions to PPD, however, did not appear until day 8 and disappeared by about day 22. They concluded that antibody-mediated pathogenesis was not of prime importance, but that the antibody might have a regulatory function.

The present work examines the possibility that B-cell immune responses may be of greater significance in the development of adjuvant disease than hitherto supposed.

MATERIALS AND METHODS

Animals. Male inbred PVG/c rats from the Allen and Hanburys colony were used in all experiments, except for those with carrageenan, in which females were used.

Drug treatment. Cyclophosphamide (Koch–Light) was administered intraperitoneally as a freshly made solution in isotonic saline.

Induction of adjuvant disease. Male rats were injected intradermally into the plantar surface of one hind paw with 0·05 ml of liquid paraffin containing 300 μg of whole heat-killed *Mycobacterium tuberculosis*, human strains C, DT and PN (MAFF, Weybridge). In the PVG/c strain, more than 95% of animals developed secondary lesions following this treatment.

The course of the disease was assessed by measuring the volume of both hind paws to the level of the calcaneum and astragalas bones, using a mercury plethysmograph connected to an air pressure transducer.

Assay for plaque-forming cells (PFC). The Cunningham slide method (Dresser & Greaves, 1973) was used to detect only direct (19S) plaques.

Immunization with sheep red blood cells (SRBC). Rats were injected intraperitoneally with 10^8 SRBC in saline. 5 days later the spleens were removed and the number of plaque-forming cells counted.

Skin allograft rejection. Tail skin from a single outbred albino rat from the Allen and Hanburys colony (Sprague–Dawley/Wistar cross) was grafted onto the thoraxes of a group of PVG/c rats. The dressings were removed after 7 days and the course of rejection followed. Tail skin isografts from a donor PVG/c rat were also performed.

Carrageenan pleurisy. The method of Di Rosa, Giroud & Willoughby (1971) was used to induce a pleurisy in rats. Female rats were injected intrapleurally with 0·15 ml of 1% Viscarin (Marine Colloids, Batch No. 7115). 6 hr later the rats were killed and 1 ml of Tyrode's solution was injected intrapleurally and the exudate recovered. The volume was measured and the total white cell counts determined using a Coulter Counter, Model ZF. Differential counts were made on Leishman-stained smears.

Delayed hypersensitivity (DH) skin tests. Rats were challenged on one flank with an intradermal injection of 50 μg of PPD (MAFF, Weybridge) in 0·1 ml Tyrode's solution. The opposite flank acted as a control site, receiving only Tyrode's solution. The sites were examined at 24 and 48 hr after challenge. Erythema was assessed as: (0) no erythema, (±) faint erythema and (+) distinct erythema. Distinct erythema was defined as a reaction in which the boundaries of the erythema were sharp enough to allow measurements of the diameter. The skinfold thickness was also measured at both sites using a Mercer micrometer. Each animal was challenged once only.

Spleen cell transfer. Individual spleens were carefully teased into cold Eagle's MEM and then sucked repeatedly into a Pasteur pipette to break up cell aggregates. The cell suspension was made up to 10 ml with Eagle's MEM and centrifuged for 2 min at 65 g to remove cell clumps. The supernatant was centrifuged at 150 g for 5 min and the resulting pellet resuspended in 1·0 ml of Eagle's MEM. 10 μl samples of this suspension were withdrawn for counting total nucleated cells with a Coulter Counter and a further 10 μl were examined by phase-contrast microscopy to give an estimate of viability (usually 60–70%). Dead cells were identified by decreased refractility and darkening of the nuclear membrane. Immediately after preparation the spleen cells were infused intravenously into recipient rats.

Collection of serum. The serum was collected from male arthritic rats 12 days after adjuvant. At this time the anti-mycobacterial antibody response is maximal (Perper *et al.*, 1972). Normal serum was collected from male rats of a similar age and both sera were stored in polystyrene tubes (Luckham) at -20°C.

RESULTS

Inhibition of adjuvant disease by single high doses of cyclophosphamide

Rats were dosed intraperitoneally with 100 mg per kg body weight Cy 3 days before the injection of adjuvant into one hind paw (in the PVG/c rat this dose is about the LD_{10}. Fig. 1 shows the volumes of both the injected and contralateral uninjected paws. Rats pretreated with Cy failed to develop the typical polyarthritis which commences in the uninjected hind paw around 14 days after adjuvant. In the injected paw, the initial lesion was only slightly inhibited, but there was marked suppression of the secondary phase of the disease, with a slowly developing but relatively mild swelling occurring by day 35. This was macroscopically different from the lesions in the arthritic rats and was thought to be a subcutaneous granuloma.

Effect of time of pretreatment with Cy on the development of arthritis

The ability to develop polyarthritis was related to the time between Cy treatment and the subsequent administration of adjuvant. The results are shown in Fig. 2. When Cy was given more than 7 days before adjuvant, the ability to develop secondary lesions reappeared, with four out of five rats responding when Cy was given 9 days before adjuvant. Recovery of normal incidence occurred when administration took

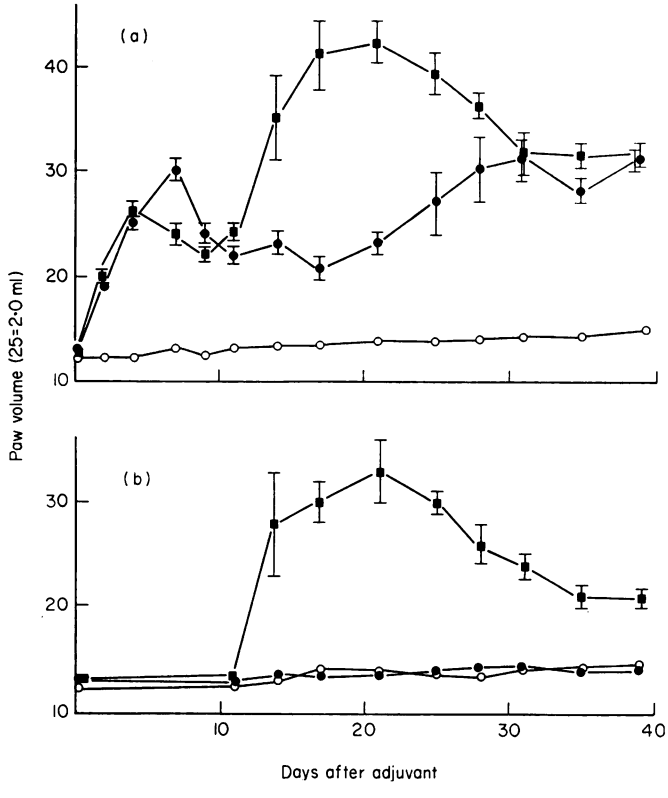

FIG. 1. Suppression of adjuvant arthritis by Cy. Results shown as mean paw volumes ± s.e.m. (not shown where it is less than 0·02 ml). (■) adjuvant only, $n=6$; (●) Cy plus adjuvant, $n=6$); (○) untreated, $n=6$. (a) Injected paw lesion; (b) contralateral paw lesion.

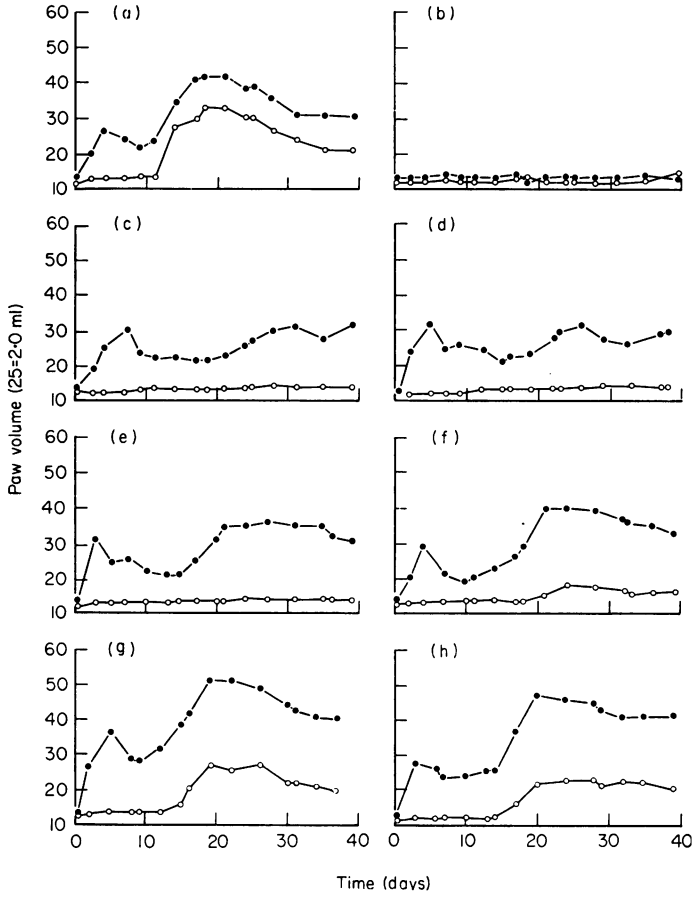

FIG. 2. Time-course of recovery of arthritic response to adjuvant in rats receiving 100 mg/kg Cy. Mean paw volumes of (●) injected paw, (○) contralateral paw. Adjuvant given on day 0. (a) adjuvant only; (b) Cy given on day 3, with no adjuvant; for other graphs Cy given on: (c) day 3; (d) day 5; (e) day 7; (f) day 10; (g) day 12; and (h) day 14 (five rats per group).

place 14 days before adjuvant. The lesion in the injected paw showed a similar time-course of recovery. One difference, however, was the apparently enhanced reaction in rats pretreated with Cy 12 or 14 days before adjuvant.

Development of delayed hypersensitivity to PPD in Cy-treated rats

Groups of rats were given 100 mg per kg of Cy intraperitoneally and 3 days later were injected with adjuvant. The animals were skin-tested with PPD 3, 7, 14, 21 and 35 days after adjuvant. Fig. 3 demonstrates that rats pretreated with Cy developed an equivalent response at days 3, 7 and 14 to those receiving only adjuvant. By day 21, the Cy group clearly showed a stronger delayed hypersensitivity (DH) reaction than the adjuvant-alone group, which had reached a peak at day 14. At day 35, Cy-treated rats still showed a significant reaction.

The erythematous component of the DH reaction was markedly intensified by Cy pretreatment. Fig. 4 shows the number of animals which developed a distinct erythema. The arthritic controls generally showed little erythema at 24 hr but usually some reaction at 48 hr. In contrast, the Cy-pretreated groups showed strongly erythematous reactions at both 24 and 48 hr. It is evident that in Cy-treated animals strong cell-mediated immune reactions to PPD can develop under conditions where the secondary lesions of arthritis are suppressed. Furthermore, Cy appears to increase the intensity of response and to prolong the sensitivity to PPD.

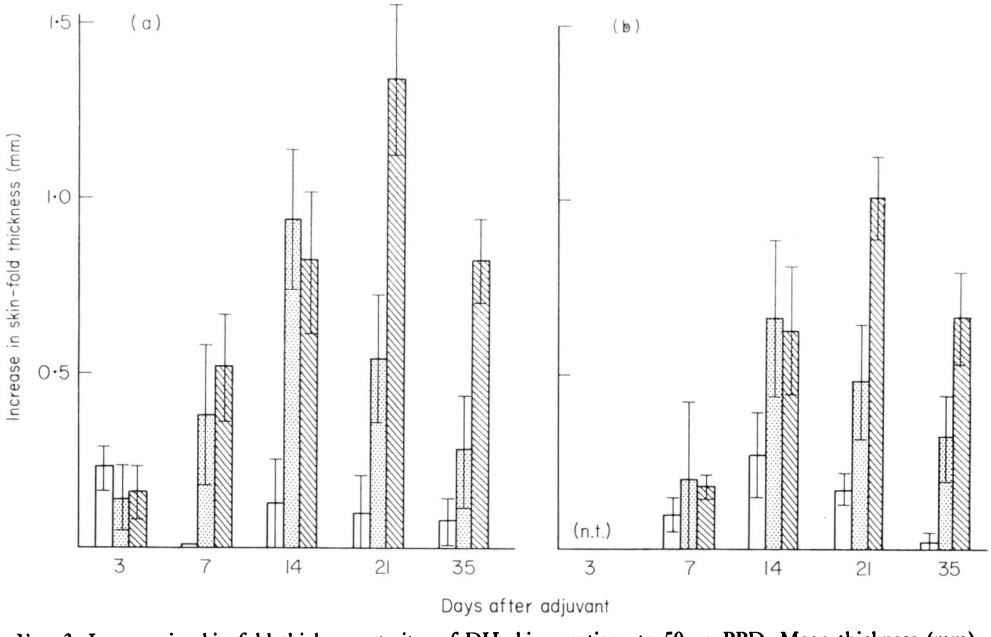

FIG. 3. Increase in skin fold thickness at sites of DH skin reactions to 50 μg PPD. Mean thickness (mm) ± s.e.m.; (□) untreated; (▨) adjuvant only; (▩) Cy plus adjuvant; (n.t.) not tested (five rats per group). (a) 24 hr; (b) 48 hr.

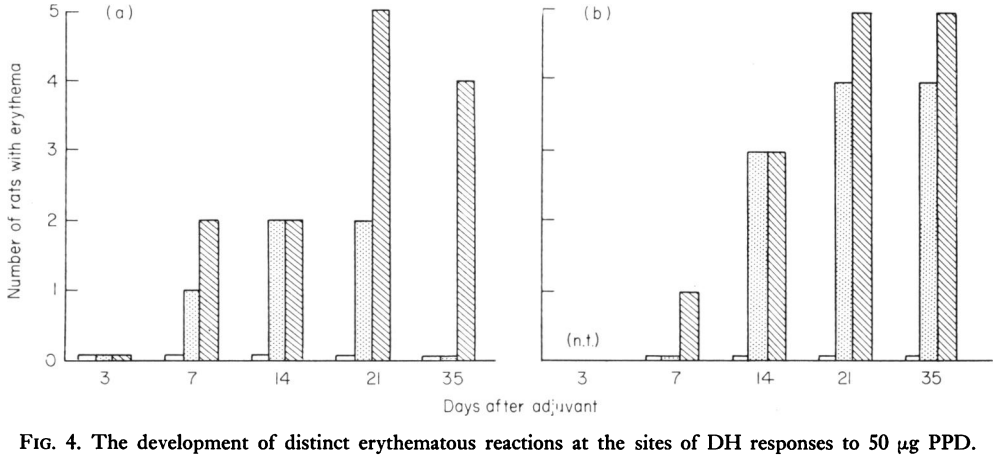

FIG. 4. The development of distinct erythematous reactions at the sites of DH responses to 50 μg PPD. Number of rats showing distinct erythema; see legend Fig. 3 for key.

Effect of cyclophosphamide dosage on adjuvant arthritis

3 days before adjuvant, rats received 12·5 to 200 mg/kg of Cy intraperitoneally. Fig. 5 shows the course of the disease in the uninjected paw. All doses except 12·5 mg/kg caused some suppression of the disease, which was totally prevented by 100 and 200 mg/kg. In this experiment the two highest doses of Cy were found to inhibit the primary lesion to some extent.

Reconstitution of Cy-suppressed rats by immune serum

Cy-pretreated rats received intravenously 0·5 ml of either neat, 1/10 or 1/50 diluted serum daily on days 0 to 5 and 7 to 11 inclusive. The first dose was given 1 hr after adjuvant. The results in Fig. 6 show that 1/50 diluted immune, but not normal, serum completely restored the arthritic reaction suppressed

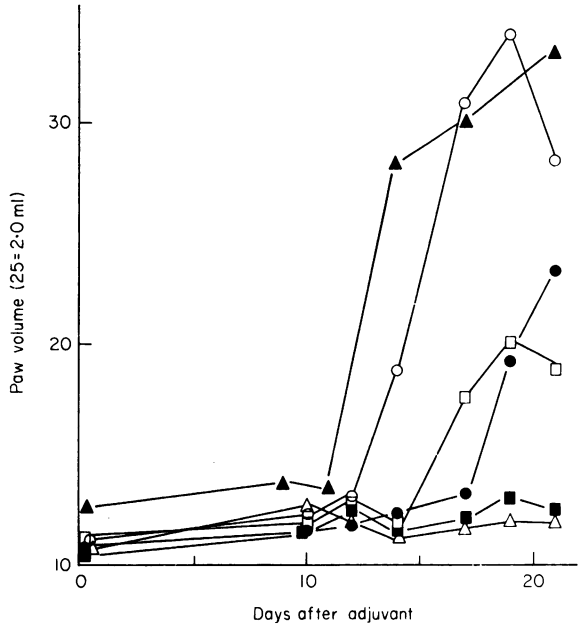

FIG. 5. Dose-dependent suppression by Cy of lesions in the uninjected hind paw in rats receiving adjuvant. Mean paw volume shown; (▲) adjuvant only; (△) 200 mg/kg Cy; (■) 100 mg/kg Cy; (□) 50 mg/kg Cy; (●) 25 mg/kg Cy; (○) 12·5 mg/kg Cy (four rats per group).

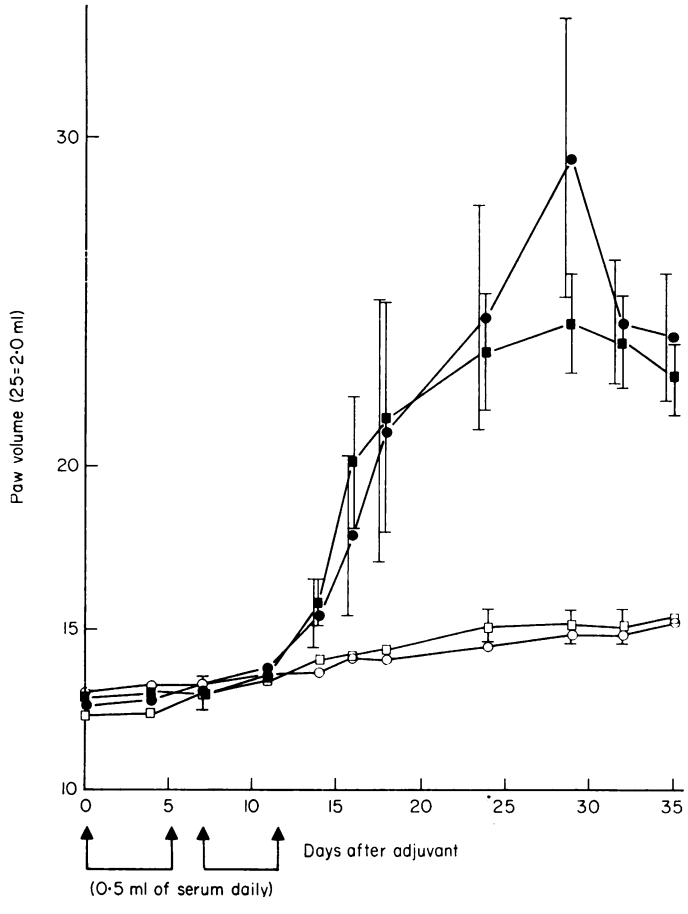

FIG. 6. Development of arthritis in Cy-pretreated rats given immune serum. Mean volume of the uninjected paw ± s.e.m. (not shown where it is less than 0·02 ml); (■) adjuvant only; (○) Cy plus adjuvant, (□) Cy plus adjuvant plus neat immune serum; (●) Cy plus adjuvant plus 1/50 diluted immune serum (five rats per group).

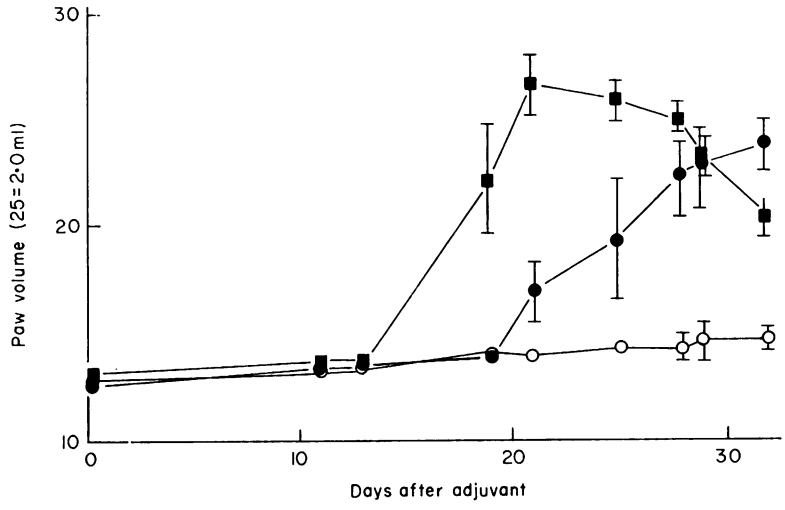

FIG. 7. Development of arthritis in Cy-pretreated rats, reconstituted with $7{\cdot}6 \times 10^7$ to $1{\cdot}5 \times 10^8$ normal spleen cells. Mean volume of the uninjected paw ± s.e.m. (not shown where it is less than 0·02 ml); (■) adjuvant only; (○) Cy plus adjuvant; (●) Cy plus spleen cells plus adjuvant (six rats per group).

by Cy. In this experiment, 1/10 diluted immune serum was ineffective and neat immune serum appeared to have a protective effect on the injected paw.

Partial reconstitution of Cy-treated rats by passive transfer of normal spleen cells

Groups of six rats were given either 100 mg/kg Cy or saline intraperitoneally 3 days before the administration of adjuvant. 2 hr before adjuvant, some animals received $7{\cdot}6 \times 10^7$ to $1{\cdot}5 \times 10^8$ viable spleen cells by intravenous infusion. The response to adjuvant was then followed over the next 32 days. Fig. 7 shows that the injection of spleen cells into rats pretreated with Cy restored the ability to develop a polyarthritis, although the appearance of secondary lesions was delayed by 1 to 2 weeks. The spleen cells appeared to have no effect on the course of adjuvant disease in rats which did not receive Cy.

Evidence that single doses of Cy depress B-cell but not T-cell immunity in the PVG/c rat

Primary response to SRBC. The number of plaque-forming cells in the spleen was compared in rats receiving either Cy or saline before antigenic challenge. Table 1 shows that the numbers of direct (IgM) plaques were suppressed below background level by 200 and 100 mg/kg Cy. Smaller doses caused a dose-dependent inhibition of the plaque number.

TABLE 1. Dose-dependent suppression by Cy of the primary antibody response of PVG/c rats to SRBC

Treatment		Log_{10} Number of direct plaque-forming Cells per Spleen (mean ± s.e.m.)
Cy dose, day 3 (mg/kg)	SRBC, day 0	
Saline	—	$1{\cdot}87 \pm 0{\cdot}05$ ($n=10$)
Saline	+	$4{\cdot}81 \pm 0{\cdot}10$ ($n=10$)
12·5	+	$4{\cdot}64 \pm 0{\cdot}10$ ($n=9$)
25·0	+	$4{\cdot}56 \pm 0{\cdot}10$ ($n=10$)
50·0	+	$3{\cdot}74 \pm 0{\cdot}25$ ($n=10$)
100·0	+	$1{\cdot}44 \pm 0{\cdot}17$ ($n=10$)
200·0	+	$< 1{\cdot}04$ ($n=9$)

TABLE 2. Mean survival time of skin isografts and allografts in normal and Cy-treated rats

Treatment	Survival time (days)	
	Isograft	Allograft
Saline	>30 (n=2)	7·92 (n=6)
100 mg/kg Cy	>30 (n=3)	8·22 (n=5)

Skin allograft rejection. The mean survival times of the grafts on rats pretreated with either 100 mg/kg Cy or saline are shown in Table 2.

Histology

Paraffin-embedded sections of lymph nodes and spleens taken 3 days after 100 mg/kg Cy were stained with haematoxylin and eosin. The lymph nodes showed a marked depletion of primary follicles, but the paracortical areas remained well-populated. In the spleen, the marginal zone of reticulum cells appeared enlarged and some shrinkage of the periarteriolar sheath was observed. The remaining part of the sheath, however, was well-populated with lymphocytes.

Effect of single high doses of Cy on acute inflammation

Since Cy may act by depleting the rat of non-lymphoid inflammatory cells, the effect of Cy on carrageenan-induced pleurisy was examined.

The results shown in Table 3 indicate that although the volume of exudate was slightly decreased, Cy markedly reduced the number of infiltrating cells.

TABLE 3. Results of three carrageenan pleurisy experiments in rats receiving either saline or Cy 3 days before carrageenan

Treatment	Exudate		PMN		Mononuclear	
	Vol±s.e.m. (ml)	Inhibition (%)	Total±s.e.m. ($\times 10^7$)	Inhibition (%)	Total±s.e.m. ($\times 10^6$)	Inhibition (%)
Saline/Carrageenan (n=16)	0·8±0·07	—	6·38±0·72	—	4·81±0·50	—
Cy/Carrageenan (n=16)	0·6±0·06	25	2·80±0·54	56	0·68±0·13	86

DISCUSSION

The secondary lesions of adjuvant-induced-disease were completely suppressed by pretreatment with 100 mg/kg of Cy 3 days before adjuvant. Turk (1973) has shown that in the guinea-pig, this regimen depresses the level of circulating B lymphocytes but leaves the T lymphocyte numbers largely unaltered. This is reflected in the lymphoid tissues by a marked depletion of B-dependent areas. A similar depletion of peripheral B lymphocytes has been observed in Cy-treated mice (Stockman *et al.*, 1973). The PVG/c rat appears to respond to Cy in a similar fashion. Histologically, B-cell depletion was seen in lymph nodes and spleen whilst T-cell areas had a relatively normal appearance. Functionally, Cy pretreatment abolished the primary antibody response to SRBC, but did not affect the skin allograft rejection time.

The evidence indicates, therefore, that suppression of adjuvant disease by Cy might involve the depletion of B lymphocytes, although the depletion of non-specific inflammatory cells might also account

for the suppression. The carrageenan pleurisy experiments show that acute inflammatory mechanisms are affected by the drug. However, this probably does not account for the suppression of adjuvant disease, since the primary adjuvant lesion was only partially suppressed and the response could be completely restored by immune serum. The duration of the suppressed state following Cy also agreed well with data reported by Stockman *et al.* (1973) showing the recovery of the spleen weight in mice following a similar treatment with Cy. Therefore, both B-dependent and T-dependent mechanisms are probably involved in the pathogenesis of adjuvant disease. That DH alone is insufficient for the development of arthritis is suggested by the enhanced DH reactions to PPD seen in Cy-pretreated rats subsequently injected with adjuvant. A similar finding was reported by Currey (1971), who found that low doses of Cy (20 mg/kg) given on days 0 to 2 after adjuvant prevented the development of arthritis, but had no effect on PPD skin tests.

The enhancement of DH reactions in Cy-treated rats may represent a true enhancement of cell-mediated immunity. Alternatively, it may be due to the abrogation of a powerful counter-irritant, i.e. adjuvant-induced polyarthritis, which would compete for available effector cells. The latter explanation seems unlikely, however, since Perper *et al.* (1972) showed that upon re-injection of adjuvant at 21 days, both the severity of arthritis and the intensity of the DH reactions increased markedly.

Whilst it is clear that PPD is not the major arthritogenic antigen (Koga & Pearson, 1973) the ability to develop DH responses to PPD after treatment with Cy suggests that the development of cell-mediated immune reactions to the other antigens in the adjuvant is unlikely to be impaired.

If B lymphocytes are involved in adjuvant arthritis, two possible mechanisms may be envisaged. There may be a direct involvement of humoral antibody or suppressor B lymphocytes may modify the cell-mediated reaction, resulting in arthritis. Since Cy-pretreated rats show enhanced DH reactions and prolonged sensitivity to PPD, it seems likely that suppressor B cells exist which depress the cell-mediated immune responses in intact rats. If this depression is so great that the elimination of antigen is inefficient, a pathological condition may result. However, since rats with enhanced DH reactions following Cy pretreatment regain the ability to develop arthritis after passive transfer of serum from arthritic rats, this is probably not the complete explanation. It is more likely that humoral antibody or immune complexes play a direct role in the pathogenesis of adjuvant disease.

The consistent inability to reconstitute the response with neat serum requires some explanation. One possibility is that there are two antagonistic effects. Immunoglobulin (or immune complexes) on the one hand may tend to cause arthritis, whilst the natural anti-inflammatory protein found in the sera of rats with adjuvant arthritis (Billingham & Gordon, 1976) may tend to prevent it. Dilution might reduce the latter to ineffective levels, but leave sufficient antibody to induce disease.

A second explanation is that the initial lesions of adjuvant arthritis may be caused by soluble immune complexes formed in antigen excess. The transfer of large amounts of immune serum would be more likely to lead to the formation of insoluble immune complexes near equivalence. These could be readily eliminated by cells of the monocyte–macrophage system. The suggestion that anti-mycobacterial antibody might be protective against adjuvant disease was made by Perper & Oronsky (1972). However, these workers did not demonstrate that the protective factor was, in fact, immunoglobulin and may therefore have been observing an effect of the anti-inflammatory protein of Billingham & Gordon (1976).

Several pieces of evidence support the view that immune complexes in antigen excess are required for the induction of adjuvant arthritis. Lennon & Byrd (1973) showed that 50% of neonatally thymectomized Lewis rats, subsequently injected with myelin basic protein and FCA, developed adjuvant arthritis. Furthermore, the severity of arthritis was inversely related to the capacity of the serum to bind ^{125}I-labelled basic protein of myelin.

Kayashima *et al.* (1976) suggested that the enhanced severity and incidence of arthritis shown in two low-responder strains of rat, following adult thymectomy, was due to the removal of suppressor T cells which normally prevented the arthritic response. However, an alternative explanation is that helper T cells are removed by thymectomy, which leads to depressed antibody synthesis resulting in a situation of antigen excess.

Dolbeare & Petersen (1973, 1974) demonstrated immune complexes by cryoprecipitation, euglobulin

precipitation and ammonium sulphate precipitation in rats developing arthritis as a secondary lesion to adjuvant-induced peritonitis. In similarly treated rats, Dolbeare & Petersen (1973) also demonstrated a rheumatoid factor-like activity in the serum.

It is interesting to note that the granulomatous reaction at the adjuvant injection site in Cy-pretreated rats, the normal arthritic reaction and the level of the DH response in Cy-pretreated rats all reach a peak at about the same time, suggesting that cell-mediated mechanisms may control the overall severity of adjuvant disease.

In view of this evidence the following hypothesis is proposed. (a) Shortly after the injection of adjuvant, small amounts of antibody to mycobacterial components are synthesized, leading to a period of antigen excess during which soluble immune complexes form. (b) These complexes allow the dissemination of mycobacterial antigen around the body and are ultimately deposited at various sites, including the joints; IgG–IgG complexes may also be involved. (c) These sites are invaded by polymorphonuclear cells (Jones & Ward, 1963), but once damage has been caused, cell-mediated mechanisms, reacting to the mycobacterial antigen derived from the trapped complexes, become the dominant reaction and determine the severity of the disease. (d) The complex-mediated damage in the joints may also lead to the creation of new antigens and the subsequent auto-sensitization reported by Berry, Willoughby & Giroud (1973).

Preliminary observations from this laboratory (Sibley, unpublished results) suggests that a similar situation may pertain in EAE in the PVG/c rat, indicating that a two-stage pathogenesis may exist in other experimental allergic diseases. It may well be that many inflammatory or autoimmune disorders, including those in man, may be dependent upon both immune complex formation and cell-mediated immunity, with the former playing an initiating role.

Thanks are extended to Professor J. L. Turk and Professor H. L. F. Currey for helpful discussions. The skilful technical assistance of Mrs P. A. Crabb is gratefully acknowledged.

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