

Effect of free radical altered IgG on allergic inflammation

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SUMMARY The rheumatoid synovium is capable of producing large amounts of IgG which may become modified by the actions of free radicals. A rat model of synovitis was established and challenged with both normal and free radical altered IgG. IgG was prepared from homologous pooled serum by high performance liquid chromatography, and free radical damage was induced by 15 minutes ultraviolet (UV) irradiation. The results showed a worsening in gross assessments of inflammation, increases in biochemical indices of lipid peroxidation, and also a rise in the proportion of IgG which, on reisolation, showed the characteristic fluorescence associated with free radical damage. This demonstrated how the presence of free radical altered IgG might convert an inflammatory insult to a more persistent stimulus, and the capacity of an environment subjected to continuing antigenic stimulation to induce further free radical damage to IgG.

Key words: rheumatoid arthritis, immune complexes, neutrophils, rats, air pouch granuloma, lipid peroxidation, iron.

The rheumatoid synovial membrane is known to be capable of synthesising immunoglobulins in similar quantities to lymph nodes¹; up to 60 mg of IgG is synthesised a day. This is an important observation in the light of recent studies showing that human IgG is susceptible to free radical modification giving a characteristic fluorescent product.²⁻⁴ Modifications of this type are believed to be a basic prerequisite for the formation of aggregates of IgG, and their subsequent reaction with rheumatoid factors⁵ leads to the deposition of immune complexes.⁶ Phagocytosis of such complexes may lead to direct tissue damage by the release of lysosomal and other tissue degrading enzymes into synovial fluid. Also, immune complex stimulation promotes a respiratory burst in neutrophils and macrophages,^{7,8} many of which are present in the rheumatoid joint. This will result in the release of oxygen derived free radicals^{9,10} in amounts sufficient to cause further tissue damage by peroxidation of membrane lipids. These peroxides may also further amplify the inflammatory response as they are chemotactic for neutrophils.¹¹

Tissue damage will also be caused indirectly after

binding of complement by immune complexes,¹² with consequent attraction of more neutrophils, increased vascular permeability, and enhanced phagocytosis. Complement levels are depleted even in early rheumatoid arthritis (RA), indicating the importance of the immune complex-complement interaction.

In this study we have used a self limiting model of allergic inflammation, the rat allergic air pouch,¹³ to test the hypothesis that autologous free radical altered, and therefore fluorescent, IgG may play an important part in the conversion of transient to persistent chronic inflammation. This model uses the delayed type hypersensitivity reaction to bovine serum albumin (BSA)¹⁴ in conjunction with an air pouch to provide suitable space for induction of the inflammatory reaction.¹⁵ This enables quantitative assessments to be made of the exudative and proliferative processes, together with biochemical analysis of the exudate and histological analysis of the pouch wall.

The inflammatory reaction in this model will resolve naturally over several weeks in the absence of repeated antigenic challenge or pro-inflammatory stimulus such as blood injection.¹⁶ The cells which line the air pouch bear a close similarity to synovial cells,¹⁷ and we therefore suggest that the allergic air pouch is an appropriate synovial model

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for the study of the potential pro-inflammatory effects of free radical altered IgG.

Materials and methods

ANIMAL MODEL

BSA allergic air pouches were induced as described by Yoshino *et al.*¹³ Briefly, male Sprague Dawley rats, obtained from Bantin and Kingman (Hull, UK), weighing 200–250 g were grouped in sixes and sensitised by intradermal injection of BSA (Sigma, Poole, UK) emulsified with Freund's complete adjuvant (Difco, West Moseley, UK). Thirteen days after sensitisation rats received a dorsal, subcutaneous injection of 8 ml air. Twenty four hours later the air pouch so formed was challenged with BSA in 2% carboxymethylcellulose (Sigma) in 0.9% saline solution with added antibiotics (benzylpenicillin (Glaxo, Greenford, UK) and streptomycin sulphate (Evans, Liverpool, UK)). To induce a more permanent inflammatory event in the air pouch animals received a further antigenic challenge (BSA in saline) five days after the initial injection of antigen into the air pouch. Animals were killed 1, 7, 14, and 28 days after the antigenic challenge or rechallenge.

PREPARATION OF AUTOLOGOUS FREE RADICAL ALTERED IgG

IgG was prepared from pooled homologous plasma by ammonium sulphate precipitation followed by purification of the precipitate containing the main immunoglobulin fraction by high performance liquid chromatography, using a TSK 3000 SW column. The protein was eluted at 1 ml/min with physiological buffer (0.067 M KH_2PO_4 +0.1 M KCl) as mobile phase. IgG was then dissolved in 40 mmol/l phosphate buffer and divided into two portions of equal volume. One half served as a control batch and received no further treatment (normal control IgG). The second portion was subjected to UV irradiation (366 nm, 254 nm source) for 15 minutes, a period insufficient to cause aggregation (free radical altered test IgG). The immunoglobulin preparation was then further diluted with physiological buffer.

INTRODUCTION OF IgG INTO AIR POUCH

BSA allergic air pouches were induced as described above. Normal or free radical altered IgG was injected into pouches in a 0.5 ml volume at the time of challenge or rechallenge. The protein content of the IgG injections (5 mg/ml) was equivalent to 1 ml injection of whole blood to parallel earlier experiments.¹⁶

ASSESSMENT OF INFLAMMATION

Animals were killed by exsanguination, their pouches were excised and drained of fluid exudate. Measurements were made of exudate volume, white cell count, and wet weight of granulation tissue. Blood and exudate were centrifuged, and samples of serum and cell free exudate were stored at -20°C .

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Samples of air pouch tissue were processed for both light and electron microscopy using standard laboratory schedules. Samples for light microscopy were fixed in formal-saline, dehydrated and paraffin wax embedded. Thin sections (5 μm) were routinely stained with haematoxylin and eosin (H & E). Deposits of ferritin and ferric iron were demonstrated by the method described by Morris *et al.*¹⁸

Frozen sections were processed to visualise IgG by an indirect immunofluorescence technique. Primary serum was rabbit antirat IgG (Miles Laboratories, High Wycombe, UK) followed by fluorescein conjugated goat antirabbit IgG (Nordic, Maidenhead, UK). Sections were then examined using UV light.

BIOCHEMICAL TESTS OF FREE RADICAL ACTIVITY

Biochemical tests were performed on cell free exudates and sera to determine their extent of lipid peroxidation and the level of free radical damage sustained by reisolated IgG. Polyunsaturated fatty acids in biological membranes are susceptible to free radical induced peroxidation to give conjugated dienes which are broken down to malondialdehyde. The degree of lipid peroxidation of biological samples can be assessed by measuring the extent of diene conjugation, and the concentration of malondialdehyde indirectly by the amount of substances reacting with thiobarbituric acid (TBA).

TBA reactivity was measured by the method of Satoh.¹⁹ Protein was precipitated from a 0.5 ml sample of cell free exudate or serum by the addition of 2.5 ml of 10% trichloroacetic acid (Fisons, Loughborough, UK). The precipitate was washed with 0.05 M sulphuric acid (BDH, Atherstone, UK) and then heated at boiling point for 30 min after the addition of 2.5 ml 0.05 M sulphuric acid (BDH) and 4 ml 0.67% TBA (Sigma) made up in 2 M sodium sulphate (Sigma). After cooling the pink chromogen was extracted with 4 ml butanol and the absorbance of the organic layer was read with a Pye Unicam scanning spectrophotometer set at 532 nm. Results are quoted as nmol of TBA reactive substances, calculated from a malondialdehyde standard curve.

Diene conjugates were measured by the method

of Lunec *et al.*²⁰ Lipids were extracted from a 0.5 ml sample of cell free exudate or serum using a mixture of chloroform and methanol (both spectroscopic grade, BDH) in the ratio 2:1. After centrifugation the absorbance of the lower organic layer at 240 nm was taken as a direct indication of the extent of diene conjugation present in the sample.

IgG was reisolated from sera and cell free exudates by high performance liquid chromatography as described above. The extent of free radical damage to IgG was assessed by measuring its total fluorescence and dividing this by its total UV absorbance, to give a ratio of damaged (fluorescent) to total (UV absorbent) IgG. This may be described as an index of free radical damage to IgG and is represented as F/UV in figures.

STATISTICAL ANALYSIS

All results are presented as the mean (SE) for each

group. The data were parametric and were analysed by Student's *t* test for significance at the 5% level.

Results

GROSS ASSESSMENTS OF INFLAMMATION (Fig. 1)

The introduction of UV irradiated IgG into the BSA allergic air pouch caused an increase in exudation and inflammatory cell infiltration after a single or two antigenic challenges when compared with normal IgG. These increases were most significant 7 to 14 days post challenge or rechallenge. After this time the number of cells and volume of exudate were significantly reduced in both test and control pouches. Fourteen days after a single antigenic challenge the wet weight of granulation tissue was significantly increased in animals receiving free radical altered IgG, though no significant differences were seen after two antigenic challenges.

SINGLE ANTIGENIC CHALLENGE

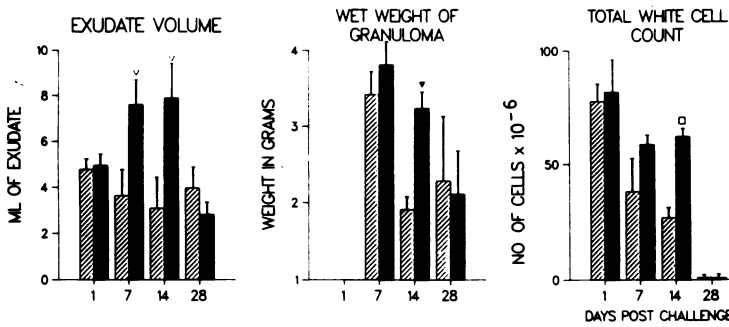
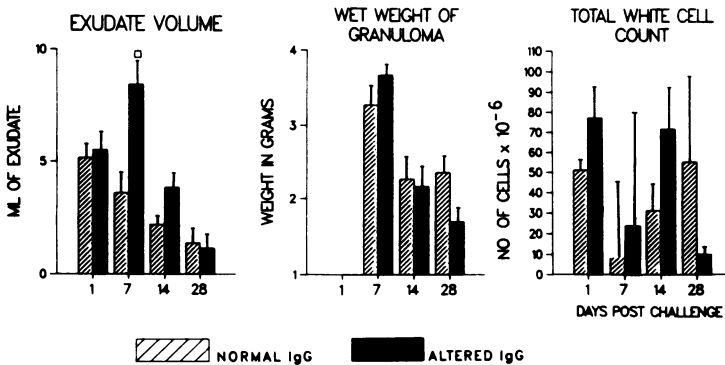
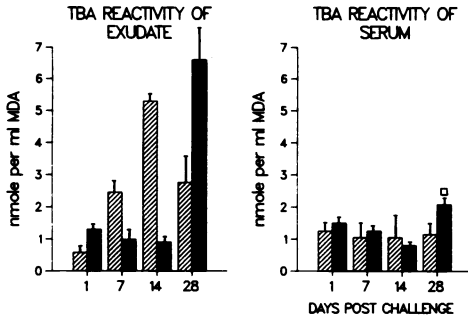


Fig. 1 Changes in exudate volume, wet weight of air pouch tissue, and total leucocyte count with time (no measurement of granulation tissue was made at day 1). ∇ = p < 0.01; □ = p < 0.05; ▼ = p < 0.001.

DOUBLE ANTIGENIC CHALLENGE



SINGLE ANTIGENIC CHALLENGE



DOUBLE ANTIGENIC CHALLENGE

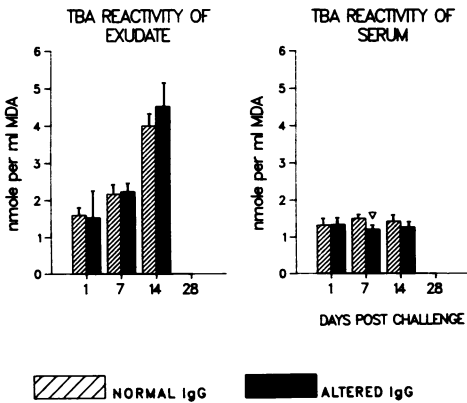


Fig. 2 Change in thiobarbituric acid (TBA) reactivity of serum and exudate with time. $\nabla = p < 0.01$; $\square = p < 0.05$. (MDA = malondialdehyde.)

BIOCHEMICAL TESTS (Figs 2 and 3)

The changes in TBA reactivity paralleled changes in the gross assessments of inflammation in that lipid peroxidation increased steadily during the experimental period. High levels of TBA reactive substances were more indicative of the chronic stages of the inflammatory reaction, being found throughout the double antigenic challenge experiment but only in the later stages of the single challenge experiment. Levels of TBA reactivity are not governed by exudate volume as, on correction for volume, the trend remained the same. TBA reactivity appears to be an indicator of local reactions of lipid peroxidation occurring within the air pouch, demonstrated by the uniform serum TBA reactivities.

The level of diene conjugates in exudates from the free radical altered IgG group was low or nil up to seven days after challenge or rechallenge. The reverse was true for serum diene conjugates, which traced a characteristic acute phase curve, the pattern of which was shifted back by five days in the double antigenic challenge experiment. In the early part of the single challenge experiment serum levels of diene conjugates were significantly higher in test animals than in control animals, though there was not such a sharp distinction between these two groups in the double challenge experiment.

Sera from rats that received free radical altered IgG after a single antigenic challenge showed little change (relative to controls) in the index of free radical damage to reisolated IgG throughout the experiment. Each of the other fluids examined from this group, i.e., exudate produced after a single antigenic challenge, and exudate and sera collected after two antigenic challenges, showed an upward trend over the 28 day experimental period. This trend was most marked in the exudate of rats which had received two antigenic challenges, though the increase over control values was not significant. The levels of fluorescent IgG were always higher in exudates than in sera, correlating with TBA reactivity results rather than diene conjugate data. The most important aspect of this result is the increase in sera of the index of free radical damage to IgG, which followed the upward trend seen in the exudate after a single antigenic challenge and injection of free radical altered IgG. This pattern of raised levels of free radical altered IgG in serum has also been seen in rheumatoid patients with high levels of free radical altered IgG in their synovial fluid.⁴

HISTOLOGY

In the single challenge experiment, pouches that had received free radical altered IgG showed an increase in cellularity and vascularity of test pouches in the early stages compared with those that had received normal IgG, the end result being a greater thickness of granulation tissue (Fig. 4).

After two antigenic challenges pouches injected with free radical altered IgG showed a highly ordered connective tissue matrix with a very active cellular lining layer (Fig. 5). Pouches challenged with normal IgG showed a barely distinguishable lining layer with a decreased thickness of organised tissue in later stages (Fig. 6).

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

(Tables 1 and 2)

Ferritin containing cells, presumably macrophages,

SINGLE ANTIGENIC CHALLENGE DOUBLE ANTIGENIC CHALLENGE

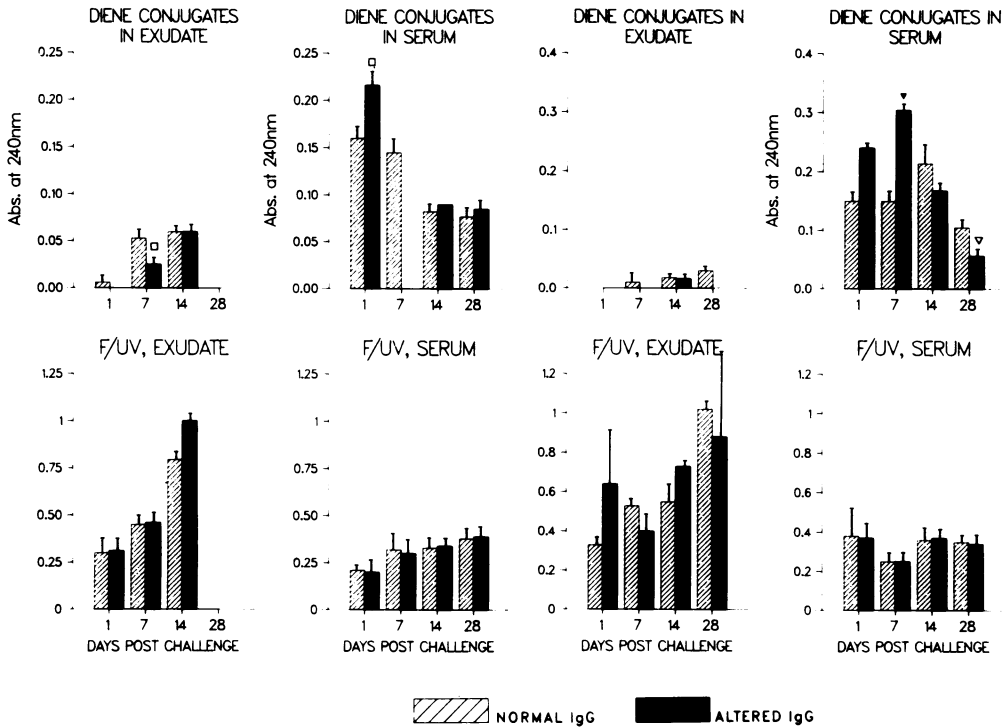


Fig. 3 Levels of diene conjugates and fluorescent (free radical damaged) IgG in serum and exudate at different stages of inflammatory reaction. ▽ = $p < 0.01$; □ = $p < 0.05$; ▼ = $p < 0.001$. (F/UV = ratio of damaged (fluorescent) to total (UV absorbent) IgG.)

were demonstrated in all sections by an immunoperoxidase technique. After a single antigenic challenge the number of positively staining cells in control pouches was significantly greater than the number in test pouches at all time points. Such positively stained cells were not associated with ferric iron, as demonstrated by Perls' technique, and were deemed to contain apoferritin.

On days 7 and 28 after two antigenic challenges the number of immunohistochemically positive cells was slightly increased, though more positive cells were found in control pouches than in those treated with free radical altered IgG. The reverse, however, was true on day 14. Cells which reacted positively to Perls' stain for iron were seen in test pouches throughout the double antigenic challenge experiment and in control pouches on day 28. The co-distribution of Perls' and ferritin staining patterns indicates the presence of iron loaded ferritin within these cells. Control tissue contained iron loaded ferritin only in the later stages of the experiment, whereas tissue taken from pouches challenged with



Fig. 4 Air pouch tissue, H & E, day 14, single challenge free radical altered IgG. Lumen of pouch at top, showing fibroblastoid cells (F), macrophages (M), active lining cell layer (L) four to five cells deep, and capillaries (C).

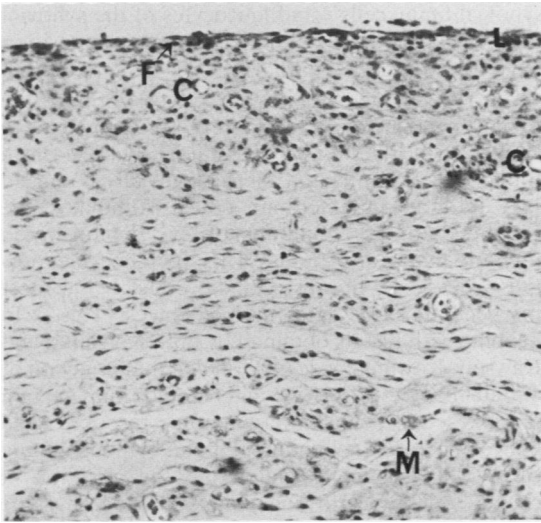


Fig. 5 Air pouch tissue, H & E, day 14, double challenge, free radical altered IgG. Lumen of pouch at top, showing fibroblastoid cells (F), foamy macrophages (M), and many capillaries (C) within organised connective tissue. The lining cell layer (L) is relatively thin.

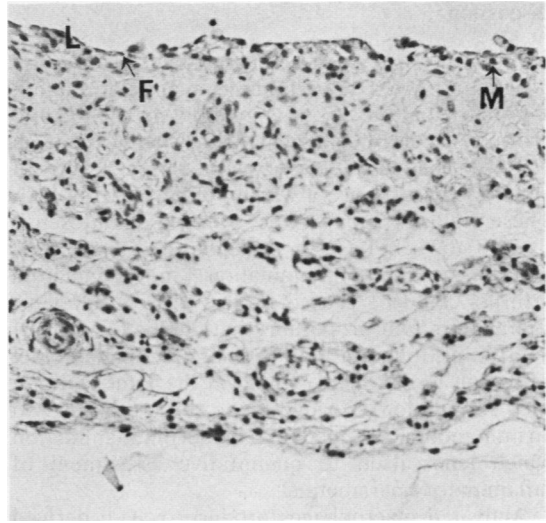


Fig. 6 Air pouch tissue, H & E, day 14, double challenge, normal IgG. Lumen at top. This resolving pouch shows reduced thickness and cellularity with macrophages (M), fibroblasts (F), and discontinuous lining cell layer (L) only two cells deep.

free radical altered IgG showed positive Perls' staining at all times after two antigenic challenges.

IMMUNOFLUORESCENCE

IgG was demonstrated in tissue from air pouches which had received two antigenic challenges. Initially (day 7), staining was restricted to test

pouches in the region of small capillaries; control pouches showed no fluorescent staining until day 14. As the inflammatory reaction progressed both the extent and intensity of the fluorescence increased until at day 28 generalised staining was seen both in pouches that had received free radical altered IgG and those that had received normal IgG.

Table 1 Numbers of air pouch cells staining positively for ferritin using an immunohistochemical technique

	Single challenge			Double challenge		
	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
NIgG†	6.8 (1.4)	11.5 (2.2)	5.3 (1.5)	8.7 (2.2)	3.6 (1.0)	7.5 (2.6)
FRIGG†	3.7 (0.6)*	5.8 (1.4)*	0*	7.8 (1.9)	6.6 (2.0)	4.9 (0.8)

Mean number (SE) of positive cells in 12 random fields.

*Statistically significant in comparison with corresponding NIgG values. $p < 0.05$.

†NIgG=normal IgG; FRIGG=free radical altered IgG.

Table 2 Numbers of air pouches staining positively with Perls' stain for ferric iron

	Single challenge			Double challenge		
	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
NIgG	0.8 (0.7)	0	0	0	0	1.4 (0.5)
FRIGG	0	0	0	4 (1.9)	1.1 (0.7)	1.3 (0.4)

Mean number (SE) of positive cells in 12 random fields.

Discussion

Several lines of evidence suggest that denaturation of IgG may give rise to immune complex formation and perpetuation of inflammation in rheumatoid arthritis, the most convincing being that virtually all of these patients present with raised levels of antibodies in their sera which are specific for IgG. These findings support the concept of auto-sensitisation to 'self IgG' in rheumatoid disease. Persistent synovial inflammation in rheumatoid arthritis could be caused by this type of antigenic stimulation. Our proposal is that the high density of neutrophils found in RA synovial fluid may interact with high levels of IgG found in the joint to cause such stimulation. To investigate this we have used an immunologically mediated model of inflammation which lends itself to quantitative assessment of inflammatory parameters.

Activated macrophages produce oxygen derived free radicals.⁷ Cell surface stimulation by particulate materials or soluble inflammatory mediators will provoke a respiratory burst in neutrophils which will also result in the production of oxygen derived radicals.²¹⁻²⁵ These highly reactive species have the potential to oxidise lipids¹¹ and denature proteins²⁶ and many other types of biomolecule²⁷. It is suggested that neutrophils and macrophages produce free radicals on stimulation, leading to damage to IgG.^{9 10}

There is increased production of IgG in rheumatoid disease,¹ but only 9% of it is found as rheumatoid factor,²⁸ the rest may have some other antigenic specificity. Fresh rheumatoid synovial fluid and sera contain fluorescent products similar to those seen on free radical damage to IgG.⁴ Rheumatoid factor has greater reactivity with denatured monomeric and aggregated IgG than with normal IgG,²⁹ so damage to IgG will lead to a greater degree of complex formation. The 15 min irradiation period used in these experiments produced mainly fluorescent monomeric denatured IgG. There was always more fluorescence in air pouch exudate than in the corresponding sera, indicating that the fluorescent IgG was probably locally produced, in contrast with the diene conjugate results for serum specimens, which appear to reflect a systemic reaction to the inflammatory insult. Diene conjugates have previously been shown to correlate with the levels of acute phase reactants in rheumatoid sera.²⁰ Here they trace a characteristic acute phase curve whose peak occurs at day 7 in the single antigenic challenge experiment and day 1 in the double challenge experiment. Immune complexes containing IgG have been demonstrated in synovial fluid^{30 31} and also within

synovial lining cells³² and leucocytes of the synovial fluid.³³ The effects of immune complex deposition have been described earlier.

Our results in the rat air pouch support the hypothesis that free radical altered IgG may have a key role in rheumatoid inflammation. We have found that the injection of free radically damaged IgG into an inflamed air pouch stimulates the production of larger amounts of IgG, demonstrated in both tissue and fluids. This IgG became progressively more fluorescent, the effect being most marked after two antigenic challenges. This is in keeping with results obtained by both Kowanko and Rawson. Kowanko *et al* have shown that injection of an air pouch with heat aggregated human IgG provokes an acute inflammatory response and phagocytosis of the immunoglobulin by pouch lining cells.³⁴ Rawson *et al* found that the injection of rheumatoid IgG into knee joints elicited an acute inflammatory response in patients with RA.³⁵ The injection of normal IgG initiated only a mild inflammatory reaction in one patient.

IgG reisolated from control pouches which had received two antigenic challenges showed a progressive increase in the formation of fluorescent IgG, though the absolute values were lower than those obtained with IgG reisolated from test pouches. This suggests that supplying a suitable IgG substrate to an environment already actively generating free radicals results in the type of chain reaction that leads to neutrophil stimulation and immune complex formation.⁴

Diene conjugates are an early indicator of lipid peroxidation; their levels fall as they undergo further peroxidation reactions to form end products that are detectable by the TBA test. Therefore our negative results may indicate one of two things; either there has been no lipid peroxidation so no diene conjugates have been formed or there has been vigorous catalysis of lipid peroxidation reactions, resulting in the conversion of diene conjugates to substances further down the peroxidative chain. The latter appears to be true in these experiments as early low values for diene conjugates were followed by high TBA reactivity values later in the experiment, indicating progressive peroxidation reactions.

In general, after two antigenic challenges the pattern of increased exudation, white cell count, TBA reactivity, and low levels of diene conjugates in exudates from pouches that had received free radical altered IgG was mirrored by control exudates. This was not the case after a single antigenic challenge when free radical altered IgG had pro-inflammatory effects compared with normal IgG.

The results therefore show how the presence of free radical damaged IgG could convert an acute inflammatory insult (single antigenic challenge) to a more persistent stimulus and also how continued antigenic stimulation (two antigenic challenges) sets up a system with enough momentum to induce damage to any normal IgG within the local environment, be it joint or air pouch.

The index of free radical damage to IgG was seen to rise in test group exudates after two antigenic challenges and this group also showed an associated rise in this index in the sera. This type of systemic effect has also been seen in rheumatoid patients who show high levels of free radical altered IgG in their synovial fluid associated with raised levels in their sera.⁴ This, together with the serum diene conjugate data, suggests that the model may be developed further to examine some of the systemic effects associated with rheumatoid disease.

Further work will examine the persistence of inflammation beyond 28 days in pouches challenged with free radical altered IgG. In an attempt to overcome the problems of low exudate volumes at this stage it is hoped that IgG may be isolated from the granulomatous pouch wall and assayed for free radical damage.

By using a naturally remitting model of inflammation, challenged with free radical altered IgG, we have a system which, we believe, mimics the type of chronic antigenic stimulation necessary for the persistence of rheumatoid inflammation.

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