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Effect of blood on the activity and persistence of antigen induced inflammation in the rat air pouch

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SUMMARY The hypothesis that haem iron derived from synovial microbleeding has a proinflammatory effect on the synovial membrane was tested by adding autologous whole blood and fractions derived from it to a naturally remitting rat air pouch model of allergic inflammation. The induction of such a subcutaneous air pouch produces a cavity lined by mesenchymal cells comparable to the synovial membrane.

Autologous whole blood was found to prolong a low grade inflammatory state, this effect being attributable to a red cell component, most probably haem iron. Whole blood in the absence of an inflammatory stimulus does not have this effect, indicating that the mechanism is one of prolonging or promoting existing allergic inflammation, rather than inducing an inflammatory response.

Rheumatoid inflammation is highly variable in its clinical manifestations. At one extreme it is a self limiting, non-destructive state, at the other a progressive locally invasive inflammatory condition. The histological appearance of the synovial membrane from both ends of this spectrum is, however, one of chronic inflammation, which in clinical terms may reflect either a transient or persistent reaction.[1] As part of a study to define what factor(s) may influence the transition between these two extremes we have suggested that traumatic microbleeding alone is a sufficient stimulus to convert chronic allergic inflammation whose 'clinical course' is transient, to a more persistent condition.

Synovial microbleeding is a recognised feature of inflammatory synovitis,[2] and both the early and established rheumatoid synovial membrane contain iron presumed to derive from degraded haem.[3] Clinical studies have suggested a relationship between the extent of iron deposition and local erosive damage which appears to be independent of the duration of the disease.[4 5] Potentially haem iron has multiple proinflammatory effects, particularly by initiating the decomposition of lipid hydroperoxides (derived from polyunsaturated fatty acids within cell membranes), which in turn will promote a chain free radical process potentially capable of disrupting cell membranes.[6]

This hypothesis has been tested by adding whole blood and fractions derived from it to an animal model of inflammation characterised by a low grade tissue mononuclear cell reaction that resolves spontaneously unless repeatedly rechallenged with antigen. The allergic air pouch model of inflammation in rats, selected and modified to provide these characteristics, has been described in detail elsewhere.[7] In brief the induction of a subcutaneous air pouch produces a membrane structure with similarities to human synovium, with a cavity lined by mesenchymal cells.[8] A single antigenic challenge to a presensitised animal with bovine serum albumin (BSA) induces acute inflammation with a predominantly polymorph infiltrate in the pouch wall and fluid in the first 48 hours; later samples show a low grade mononuclear response settling over five days. Repeated antigenic challenge induces more permanent chronic inflammation with an accentuated mononuclear response but this still recedes, settling over a 14-day period after the final challenge. The current study looks at the effect of autologous blood on the persistence of this model of transient but chronic inflammation.

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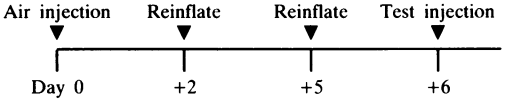
Materials and methods

1. INDUCTION OF AIR POUCH WITHOUT ALLERGIC INFLAMMATION



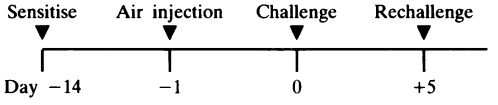
An air pouch was formed on the dorsum of male Sprague-Dawley rats (150–200 g) as described previously.[8] In brief 20 ml of air was injected subcutaneously on the dorsum of the rat on day 0, and the developing air pouch was reinflated with 15 ml of air on days +2 and +5.

2. EFFECTS OF HAEM ON NON-ALLERGIC INFLAMMATION

A. Single whole blood injections

The effects of injections of 0.25, 0.5, and 1 ml of autologous whole blood were studied in the non-allergic model. Blood or a saline (0·15 M sodium chloride) control was injected into the well formed air pouch on day +6, and animals were killed on day +7 and day +20.

3. INDUCTION OF AIR POUCH WITH ALLERGIC INFLAMMATION



Male Sprague-Dawley rats were sensitised to bovine serum albumin (BSA) emulsified with Freund's complete adjuvant on day −14 as described previously.[7] An air pouch was formed at day −1, and 24 hours later (day 0) an acute inflammatory response was induced in the pouch by a challenge with BSA in a solution of sodium carboxymethyl cellulose in saline supplemented with antibiotics. A more permanent chronic inflammatory response was induced by a rechallenge with BSA in saline on day +5.

4. EFFECTS OF HAEM ON ACUTE AND CHRONIC ALLERGIC INFLAMMATION

A. Single whole blood injections

The effects of a single injection of 0·25, 0·5, or 1 ml of autologous whole blood or an equivalent volume of 0·15 M saline injected into the air pouch were studied in both the acute phase immediately after the first injection and in the chronic phase 14 days after a second antigenic challenge. In the acute model blood was injected immediately after the antigenic challenge on day 0, and animals were killed on day +14. In the chronic model blood was injected after the rechallenge on day +5 and animals were killed 14 days later (day +19).

B. Multiple whole blood injections

In further groups of rats the effects of multiple doses of autologous whole blood on the persistence of the response was studied to see: (1) how long blood alone could prolong pre-existing allergic inflammation; (2) whether repeated bleeding in the form of multiple blood injections had a cumulative effect. In study (1) a single fixed volume (1 ml) of whole blood or saline control was injected into the air pouch immediately after the antigenic (Ag) challenge on days 0 (single challenge) or 5 (repeat Ag challenge). Animals were killed 1, 7, 14, and 28 days after the blood injection in both the single challenge (days +1, +7, +14, and +28) and repeat challenge (days +6, +12, +19, and +33) experiment. In study (2) air pouches in all rats were injected with antigen on day 0, and the animals were divided into three equal groups designated A, B, and C. 50% of animals in group A (controls) received air pouch injections of 0·5 ml of saline, and the remaining animals in group A and all in B and C received 0·5 ml of autologous whole blood. These injections (without antigen) were repeated on days +7 and +14. On day +21 all rats in group A were killed, and animals in group B and C were rechallenged with antigen. Immediately afterwards 50% of the rats in group B were injected with 0·5 ml of saline, all the others in groups B and C received 0·5 ml of whole blood, and the injection schedule was repeated (without antigen) on days +28 and +35. Animals in group B were killed on day +42 and those in group C were rechallenged with antigen and injected with saline or blood as before. Control or blood injections were repeated on days +49, +56, and +63. These rats were killed on day 70.

C. Injection of blood components

Plasma, red cells, haemolysate, and red cell membranes were added to the acute and chronic air pouch to establish which component(s) maximally affected the inflammatory response. 1 ml of each blood component was injected into the air pouch at the time of the first (day 0) or second (day 5) antigenic challenge. A group of rats was killed eight days after the initial challenge (day 8) and the second antigenic challenge (day 13) respectively.

5. ASSESSMENT OF INFLAMMATION

The following assessments of the air pouch response were made at the termination of each experiment as described previously:[7] (1) the total exudate volume;

(2) the leucocyte count in the exudate; and (3) the degree of tissue response as assessed directly by weighing the excised pouch wall.

6. PREPARATION OF AUTOLOGOUS WHOLE BLOOD AND BLOOD COMPONENTS

1 ml of blood was withdrawn from the rat's tail vein into a syringe treated with 2·5% trisodium citrate solution, and thereafter handled at 4°C. In the study of blood components (section 4C) plasma and blood cells were separated by centrifugation (1500 *g* for 10 min) and white cells removed. A haemolysate was prepared from the blood cells washed three times with 0·15 M sodium chloride and lysed hypotonically with distilled water. Cell membranes were removed by centrifugation and isotonicity was restored with sodium chloride. An appropriate dose of trisodium citrate solution was added to these blood components.

7. STATISTICAL ANALYSIS

All experiments described were performed using male rats in groups of five. The data were parametric and were analysed by Student's *t* test.

Results

Section numbers correspond to those in 'Materials and methods'.

ADDITION OF WHOLE BLOOD TO AN UNINFLAMED AIR POUCH (Table 1; section 2A)

Addition of variable doses of whole blood up to 1 ml did not induce an exudative or cellular response within the air pouch cavity, as assessed at two or 14 days after the addition of blood. There was an increase in granulation tissue shown by a progressive increase in pouch wall weight with increasing volumes of blood, though this was not totally significant.

EFFECT OF VARYING DOSES OF BLOOD IN THE ALLERGIC AIR POUCH (Table 2; section 4A)

Addition of whole blood showed a clear dose response augmenting both the acute and chronic phases of this model. There was an increase in both the volume and the leucocyte content of the exudate and of the granulation tissue wet weight, which was statistically significant.

DURATION OF RESPONSE AFTER BLOOD INJECTIONS IN THE ALLERGIC AIR POUCH (Table 3; section 4B, study (1))

Addition of a fixed volume (1 ml) of blood to the inflamed air pouch showed that the duration and the extent of the exudative and cellular response were increased more than twofold and that this was more pronounced in animals who had received a second

Table 1 *Effect of varying doses of whole blood on the uninflamed air pouch*

Blood (ml)	*Day +7*			*Day +20*		
	Granulation tissue wet wt (g)	*Exudate volume (ml)*	*No of cells × 10^{-7}*	*Granulation tissue wet wt (g)*	*Exudate volume (ml)*	*No of cells × 10^{-7}*
0	1·77±0·12	0	0	1·31±0·12	0	0
0·25	1·82±0·12	0	0	1·27±0·10	0	0
0·5	2·10±0·09	0	0	1·23±0·15	0	0
1·0	2·19±0·16	0	0	1·42±0·17	0	0

Variable doses of blood (saline) added to a six-day air pouch (see 'Materials and methods'). Assessments made one and 14 days later. Values are the means ±SEM of five rats. Significant differences were not observed.

Table 2 *Effect of varying doses of whole blood on antigen induced inflammation in the rat air pouch*

Blood (ml)	*Acute phase (first challenge)*			*Chronic phase (second challenge)*		
	Exudate volume (ml)	*No of cells × 10^{-7}*	*Granulation tissue wet wt (g)*	*Exudate volume (ml)*	*No of cells × 10^{-7}*	*Granulation tissue wet wt (g)*
0 (saline)	0	0	1·34±0·08	0	0	1·36±0·07
0·25	7·26±0·40*	2·33±0·15*	1·78±0·12*	1·05±0·48	0·41±0·17	1·48±0·06
0·5	9·27±0·67*	3·62±0·25*	2·03±0·12*	3·17±0·35*	1·28±0·13*	1·89±0·12*
1·0	11·03±0·61*	4·56±0·29*	2·18±0·14*	9·34±0·56*	2·43±0·27*	2·03±0·16*

The values are the means ±SEM of five rats.
* Statistically significant compared with corresponding values of 0·15 M NaCl (saline) group: $p<0·01$.

antigenic challenge at day 5. In both cases the blood was injected into the air pouch immediately after the final antigen dose.

ADDITION OF MULTIPLE DOSES OF WHOLE BLOOD TO THE ALLERGIC AIR POUCH (Table 4; section 4B, study (2))

These experiments showed that the effect of multiple doses of blood was cumulative with progressive increases in both the exudative, cellular, and tissue response. This was particularly seen in the tissue response in the groups given six and 10 injections. However, as illustrated in the relevant control experiments these proinflammatory effects could only be maintained by the continued addition of whole blood; six injections of blood when followed by four saline injections were associated with resolution of the exudative, cellular, and tissue inflammatory responses.

ADDITION OF BLOOD COMPONENTS TO THE ALLERGIC AIR POUCH (Table 5; section 4C)

After a single injection blood cells had a proinflammatory effect on both the exudate (volume and cell number) and the granulation tissue wet weight as potent as that of whole blood. The red cell haemolysate was almost equally potent, but plasma and cell membranes produced little effect. After a second antigenic challenge the single dose of blood cells and haemolysate produced an equivalent response, but the plasma factor also contributed a proinflammatory effect which was most pronounced on the exudate volume and cell numbers, with no statistical effect on granulation tissue wet weight.

Table 3 *Time course of the effect of blood on antigen induced inflammation in the rat air pouch*

Day	*Group*	*Acute phase (first challenge)*			*Day*	*Chronic phase (second challenge)*		
		Exudate volume (ml)	*No of cells* $\times 10^{-8}$	*Granulation tissue wet wt (g)*		*Exudate volume (ml)*	*No of cells* $\times 10^{-8}$	*Granulation tissue wet wt (g)*
+1	Saline	6·97±0·37	1·97±0·07	—	+6	7·85±0·54	2·35±0·14	—
	Blood	10·00±0·31*	2·54±0·10*	—		11·04±0·75*	3·35±0·21*	—
+7	Saline	5·72±0·47	0·27±0·03	1·53±0·08	+12	7·05±0·46	0·17±0·02	2·10±0·08
	Blood	12·81±0·54*	0·49±0·02*	2·23±0·10*		12·20±0·88*	0·39±0·02*	2·75±0·04*
+14	Saline	0	0	1·45±0·04	+19	0	0	1·67±0·09
	Blood	10·79±0·74*	0·45±0·02*	2·01±0·07*		10·18±0·99*	0·28±0·03*	2·78±0·07*
+28	Saline	0	0	1·15±0·06	+33	0	0	0·77±0·05
	Blood	1·58±1·58	0·02±0·02	1·77±0·06*		4·78±0·64*	0·12±0·01*	1·66±0·08*

Numerical values are the means ±SEM of five rats.
* Statistical significance: $p<0·01$.

Table 4 *Effect of repeated injections of autologous blood on antigen induced chronic inflammation in the rat air pouch*

	Weekly injections of blood (or saline)	*Exudate volume (ml)*	*No. of leucocytes* $\times 10^{-7}$	*Granulation tissue wet wt*[9]
A	(3)	0	0	1·30±0·10
	3	7·25±0·59*	0·46±0·06	1·94±0·18*
B	3 + (3)	0	0	1·40±0·08
	6	9·18±0·57*	0·79±0·09*	2·12±0·10*
C	6 + (4)	0	0	1·02±0·14
	10	15·38±1·97*	1·17±0·13*	2·38±0·20*

All animals were sensitised to BSA (day −14), an air pouch was formed (day −1) and an antigenic challenge given at day/week 0. Animals were divided into three groups (A–C). Group A did not receive a further antigenic challenge, group B received another challenge at week 3, and group C, two challenges at weeks 3 and 6. Each group was subdivided into those receiving 0·5 ml of blood or saline or blood followed by saline. Blood (saline) was given at weekly intervals starting at week 0, as indicated in the table. Weeks unbracketed indicate the number of consecutive weekly injections of blood. Weeks bracketed correspond to saline injections (e.g. 6 + (4) indicates six injections of blood followed by four of saline). All animals were killed seven days after the last injection. Assessments as indicated in text.
Values are means ±SEM of five rats.
* Significant when compared with values of control $p<0·01$.

Table 5 *Effect of blood components on acute and chronic inflammation*

Group	*Acute*			*Chronic*		
	Exudate volume (ml)	*No of cells × 10^{-7}*	*Granulation tissue wet wt (g)*	*Exudate volume (ml)*	*No of cells × 10^{-7}*	*Granulation tissue wet wt (g)*
Saline	1·00±0·63	0·15±0·10	1·60±0·06	2·31±0·41	0·03±0·01	1·63±0·05
Whole blood	11·58±0·95*	2·80±0·22*	2·26±0·11*	9·14±0·52*	0·21±0·02*	2·14±0·12*
Blood cells	10·22±0·76*	2·26±0·18*	2·18±0·15*	8·66±0·55*	0·19±0·02*	1·99±0·09*
Haemolysate	7·56±0·46*	1·83±0·21*	2·05±0·07*	7·20±0·16*	0·16±0·01*	1·95±0·04*
Plasma	2·42±0·76	0·32±0·11	1·80±0·12	5·59±0·27*	0·10±0·01*	1·81±0·04
Cell membranes	2·01±0·59	0·26±0·12	1·74±0·10	3·57±0·30	0·05±0·01	1·70±0·04

1 ml each indicated blood component was injected into the pouch immediately after the antigenic challenge (acute) and rechallenge (chronic). Eight days after each challenge the animals were killed and the volume of exudate, the number of white cells in the exudate, and the wet weight of granulation tissue were measured.
The values are the means ±SEM of five rats.
* Statistically significant when compared with values of saline groups: $p<0·01$.

Discussion

The model of antigen induced inflammation in the rat air pouch is an example of a connective tissue activation and granulation tissue formation in response to immunological stimuli. It differs from other experimental models of chronic synovitis such as the Dumonde-Glynn rabbit model, or the adjuvant or collagen rat arthritis, in its natural tendency to resolve without repeated antigenic challenge.[7] In this respect this model is much closer to the human rheumatoid arthritis pattern of natural flares and remissions. Although it is not a model of synovitis, Edwards and his colleagues have shown that the cell types which line this artificial gap in the connective tissue bear a close similarity to those seen in the synovium.[8]

We have shown in this simple series of experiments that the addition of autologous whole blood to such a naturally remitting allergic model of inflammation prolongs a low grade inflammatory state. The effect is mainly attributable to a component within the red cell. Whole blood in the absence of an inflammatory stimulus has no such proinflammatory activity; the mechanism therefore appears to be one of promoting/prolonging allergic inflammation rather than inducing inflammation.

Further work is in progress to define precisely what factor in the red cell induces this phenomenon, but in-vivo studies suggest that the most likely contender is haem iron. Muirden was one of the first to suggest that rheumatoid synovitis might be exacerbated by synovial iron deposits,[4] and it has been recently shown that patients with early rheumatoid synovitis with significant deposits of iron within their macrophages tend to have a persistent disease.[5] A variety of mechanisms have been proposed which might explain the phenomenon. Ferrous iron will activate the formation of the toxic hydroxyl radical from hydrogen peroxide, which may then promote the production of organic oxygen radicals and lipid peroxides from polyunsaturated fatty acids.[6 9] Haem iron will produce lipid peroxidation directly.[9] Rowley *et al.* have confirmed that lipid peroxidation products are present in rheumatoid synovial fluid and correlate with the extent of the inflammatory reactions.[10] Ionic ferric iron, or iron chelates stimulate the division of rabbit synovial fibroblasts and in addition lead to the release of the prostaglandin PGE_2 and the enzyme collagenase.[11]

Our results in the air pouch support the hypothesis that the continued microbleeding which occurs in rheumatoid synovitis is a factor in the persistence of this disease. These results supplemented by the in-vitro observations suggest that many of the well documented clinical and laboratory features of rheumatoid disease could be explained by repeated haem promoted exacerbation of an allergic synovitis. This hypothesis would explain both the persistence of the condition and the established clinical observation of the beneficial effect of bed rest. The latter which would minimise traumatic microbleeding remains one of the most effective but previously unexplained means of suppressing synovial rheumatoid inflammation.

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References

1 Norton W L, Ziff M. Electron microscopic observations on the rheumatoid synovial membrane. *Arthritis Rheum* 1966; **9:** 589–610.

2 Bennett R N, Williams E D, Lewis S M, Holt P J L. Synovial iron deposition in rheumatoid arthritis. *Arthritis Rheum* 1976; **16:** 298–304.

3 Muirden K D, Fraser J R E, Clarris B. Ferritin formation by synovial cells exposed to haemoglobin in vitro. *Ann Rheum Dis* 1967; **26:** 251–9.

4 Muirden K D. Ferritin in synovial cells in patients with rheumatoid arthritis. *Ann Rheum Dis* 1966; **25:** 387–401.

5 Blake D R, Gallagher P J, Potter A R, Bell M J, Bacon P A. The effect of synovial iron on the progression of rheumatoid disease. A histologic assessment of patients with early rheumatoid synovitis. *Arthritis Rheum* 1984; **27:** 495–501.

6 Blake D R, Hall N D, Bacon P A, Dieppe P A, Halliwell B, Gutteridge J M C. The importance of iron in rheumatoid disease. *Lancet* 1981; **ii:** 1142–4.

7 Yoshino S, Bacon P A, Blake D R, Scott D L, Wainwright A L, Walton K W. A model of persistent antigen induced chronic inflammation in the rat air pouch. *Br J Exp Pathol* 1984; **65:** 201–14.

8 Edwards J C W, Sedgwick A D, Willoughby D A. The formation of a structure with the features of synovial lining by subcutaneous injection of air. An in-vivo tissue culture system. *J Pathol* 1981; **134:** 147–56.

9 Blake D R, Lunec J, Winyard P, Brailsford S. Iron and inflammation. In: Sturrock R, ed. *Recent advances in rheumatology 3.* Edinburgh: Churchill Livingstone, in press.

10 Rowley D, Gutteridge J M C, Blake D R, Farr M, Halliwell B. Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in synovial fluid from rheumatoid arthritis. *Clin Sci* 1984; **66:** 691–5.

11 Okasaki I, Brinckerhoff C E, Sinclair P R, Bronkowsky H L, Harris E D. Iron increases collagenase production by rabbit synovial fibroblasts. *J Lab Clin Med* 1981; **97:** 396–402.

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