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The subcutaneous air pouch model of synovium and the inflammatory response to heat aggregated gammaglobulin

INGEBORG C. KOWANKO[1], THOMAS P. GORDON[1], MICHAEL A.M. ROZENBILDS[3], PETER M. BROOKS[1], AND PETER J. ROBERTS-THOMSON[2]

[1] Rheumatology Research Group, Departments of Medicine, [2] Clinical Immunology and [3] Pathology, Flinders Medical Centre, Bedford Park, South Australia, 5042

Summary

Subcutaneous injection of sterile air in rodents results in the formation of an air pouch with a lining morphologically similar to synovium (Edwards et al., 1981) [1]. We extended the comparison between pouch and synovial tissue and confirmed broad similarities in structure and function but also noted important differences. The air pouch was used to study the time course of the acute inflammatory response to heat aggregated human IgG. Saline washout of the pouch allowed simultaneous measurement of cellular and mediator components of the inflammatory exudate. The aggregates were rapidly phagocytosed by the pouch lining cells, resulting in acute inflammation characterised by polymorphonuclear leucocyte infiltration with peak numbers in the exudate at 12 hours, temporally dissociated from the earlier peak of PGE_2 at 3 hours.

Introduction

Injection of air subcutaneously in the rat and mouse results in the formation of an air pouch. Selye [2] studied inflammation in the pouch by injecting phlogistic agents at the time of pouch formation to form a granuloma pouch. This model was subsequently used in the study of anti-inflammatory agents [3–5] and infection [6]. Recently Edwards et al. [1] observed that if air pouches were kept inflated by the repeated injection of air alone then they developed a lining with the appearance of synovium. This group went on to use such 'simple' air pouches as models of the synovial cavity [7, 8]. In this paper we describe our own comparisons between the structure and function of the air pouch and synovium which confirm and extend the findings of Edwards et al. Important differences are also noted.

The subcutaneous air pouch appears more like a bursal cavity than a synovial joint. Nevertheless we believe that the air pouch is a suitable model in which to study aspects of the acute inflammatory response to agents implicated in synovial joint disease. We can monitor the cellular and mediator components of the exudate by saline washout, as well as examining the histopathology of the inflamed tissue [9]. We have used the air pouch model to investigate the mechanism of acute inflammatory responses to monosodium urate crystals [10] and model immune complexes (IC) [11].

IC-mediated inflammation warrants study because there is much evidence that IC are pathogenic in rheumatoid arthritis (RA) and other rheumatic diseases. IC are found in high concentrations in the synovial fluid of many patients with RA [12] and are trapped preferentially in the collagenous joint tissues of naturally occurring and experimental arthritis [13, 14]. Heat aggregated human gammaglobulin (HAGG), used as model IC, induced acute inflammation when injected into the uninvolved knee joints of patients with RA [15]. Similarly injection of HAGG or IC into rabbit knees resulted in synovitis [16]. There is also *in vitro* evidence that both HAGG and preformed IC activate neutrophils directly [17].

We present here studies on the acute inflammatory response to HAGG in the air pouch. The time course and possible mechanism of inflammation are discussed.

Correspondence to I. C. Kowanko, present address: Department of Paediatrics, Adelaide Children's Hospital, North Adelaide, South Australia 5006.
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Materials and methods

The first part of the study was designed to test the validity of the air pouch as a model of synovium. Rats were killed at weekly intervals for 7 weeks after air pouch formation and paired pouch and synovial sections and saline washouts of the pouch examined using light and electron microscopy. The functional similarity of the pouch to synovium was investigated by studying phagocytosis and the ability to mount acute and chronic inflammatory responses.

The second part of the study used the air pouch to investigate the mechanisms of the acute inflammatory response to HAGG (model IC).

Air pouch formation. Male Hooded Wistar or J. C. Lewis strain rats weighing 200-300g were anaesthetised with ether and their backs shaved and swabbed with alcohol. Twenty ml of air, sterilised by passage through a 0.2 μ filter, was injected subcutaneously to form the pouch. Pouches were reinflated twice weekly with 5-10 ml air to maintain inflation.

Washouts of the pouch were made by deflating the pouch, irrigating with 5 ml normal saline and withdrawing 4 ml. Cell counts were made using a haemocytometer or Coulter counter. Differential counts were performed manually on Giemsa stained cytocentrifuge preparations.

Phagocytic ability of the pouch and synovium was assessed by injecting colloidal carbon as 2 ml of sterile 0.1% shellac-free India ink (Pelikan) into the 7 day old pouch, or 0.05 ml into the knee jont. Six hours later pouch lining and synovium were removed for histology.

Acute inflammatory responses were elicited by injecting irritants into the 7 day old air pouch cavity. Irritants used were carrageenan type 7 (Marine Colloids, Springfield, New Jersey. Lot 282104) (10 mg in 1 ml normal saline), carrageenan type 10 (Seachem Co., Maine), oleyl alcohol (1 ml of a 10% aqueous suspension), killed *Mycobacterium tuberculosis* (0.5 mg in 1 ml sesame oil) and suspensions of monosodium urate crystals (15 mg in 2.5 ml phosphate buffered saline), calcium pyrophosphate dihydrate crystals (15 mg in 2.5 ml normal saline) and calcium hydroxy apatite crystals (15 mg in 2.5 ml normal saline). The histology of the pouches at 24 hours was assessed.

Chronic inflammatory responsiveness of the pouch to systemically injected adjuvant was assessed. Twenty-four rats were injected with 0.05 ml of 10 mg/ml emulsion of heat-killed delipidated *M. tuberculosis* cells in squalane (adjuvant) into the base of the tail. 10-14 days later the rats developed polyarthritis which was monitored by measuring weight loss, hindpaw swelling and vasculitic lesions for an arthritis score. The rats were killed 14 days after adjuvant injection. Saline washouts of the pouch were made and knee synovia removed for histology. Other rats were killed at 2, 4, 5, 7, and 11 days after adjuvant injection to assess the development of the disease. Samples of connective tissue from beneath the dorsal skin remote from the pouch, peritoneal and pleural membranes were also screened for the presence of inflammatory cells.

Histology. Rats were sacrificed and the air pouch and synovial tissue rapidly removed and transferred to glutaraldehyde-formaldehyde fixative or snap frozen.

Paraffin sections were stained with haematoxylin and eosin (H + E) for light microscopy. Resin embedded sections were prepared for electron microscopy by post-fixing in osmium tetroxide and staining with uranyl acetate and lead citrate.

Macrophage-like cells in the air pouch lining and synovium were characterised using histochemical and immunohistological markers. The lysosomal enzyme tartrate-resistant acid phosphatase was demonstrated using a simultaneous capture method with naphthol ASBI phosphate substrate and hexazonium pararosanilin dye at pH = 5. Lysosomal non-specific esterase was demonstrated using a similar method with naphthyl acetate as a substrate and sodium fluoride inhibition. The macrophage markers muramidase, α-1-antitrypsin and α-1-anti chymotrypsin were demonstrated using a peroxidase-antiperoxidase technique with anti-human antibodies.

Frozen sections were treated with a panel of monoclonal antibodies (Seralab) to rat cell surface antigens using an immunoperoxidase method. Antibodies used were OX-1 (anti leucocyte), OX-3 and OX-6 (anti Ia surface antigens). Sections of pouch with HAGG-induced inflammation were routinely stained with H + E, and also with fluorescein-labelled anti-human IgG to demonstrate uptake of HAGG.

Monosodium urate crystal treated pouches were fixed in absolute alcohol and stained with methenium silver.

HAGG-indicated inflammation was initiated by deflating lin (Cohn fraction 2, CSL) in phosphate buffered saline (PBS) to 20 mg/ml and heating at 63°C for 55 mins. 5 ml aliquots were stored at − 20°C until used.

Hagg-induced inflammation was initiated by deflating rat air pouches made 7 days previously and injecting 1 ml of HAGG solution (20 mg/ml) into the cavity. Unaggregated IgG (20 mg/ml) or PBS served as controls. The time course of the response to HAGG or control was studied by sacrificing rats at 1.5, 3, 6, 12 and 24 hours after HAGG injection, washing out the pouch with 5 ml saline and measuring white cell counts and differential counts. Supernatants were stored at −20°C for later assay of prostanoid mediators and lysosomal enzymes. Samples of pouch were taken for histology and vascular permeability changes were assessed as described below. The response to different doses of HAGG was also studied.

Prostanoid mediators. The prostaglandins PGE_2 and 6-keto $PGF_{1\alpha}$ (the stable metabolite of prostacyclin), and the lipoxygenase pathway product 5-HETE were measured by radioimmunoassay after ether extraction of the washout.

Lysosomal enzymes measured were β-glucuronidase, β-glucosidase, acid phosphatase and β-hexosaminidase using a fluorimetric method.

Vascular permeability was studied at 15, 30, 45 mins and 1.5, 3 and 6 hours after HAGG or PBS injection. Rats were injected i.v. with 1 ml of 50 mg/ml fluorescein labelled bovine serum albumin as a marker of the vascular space [18] 5 mins before sacrifice. Pouch tissues were fixed in formalin and sections were viewed in the wax with a fluorescence microscope. The appearance of fluorescent material in the interstitial tissues near venules indicated increased permeability. The method was verified by demonstrating vascular leakage 10 mins after histamine intradermally.

Results

Morphology of non-inflamed pouch lining and synovium

The interior surface of the air pouch was smooth and glistening with a fibrous stromal structure containing 4-10 layers of cells of mainly spindled shape. The pouch was established within

a week of formation and could be maintained for 50 days with repeated injection of sterile air. The wall of the pouch became progressively thicker with time, up to 20 cells deep. Seven days after formation the pouch resembled a bursal cavity with a lining superficially like synovium, but with minimal fluid exudate adherent to the wall.

Light microscopy revealed that, like synovium, the inner lining of the air pouch had a surface of flattened or spindled cells (Fig. 1).

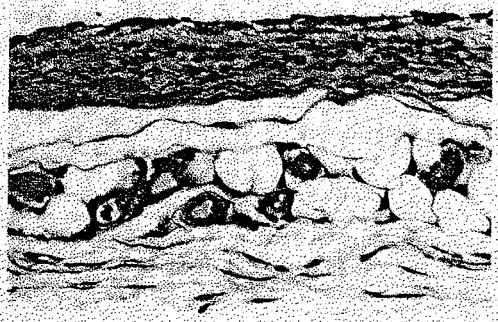

Figure 1
Unstimulated air pouch tissue, 7 days after formation, showing flattened cells lining the cavity (top) overlying vascular connective tissue. H + E, X 480.

Electron microscopy showed that the cells lining the pouch were of 2 main types: a fibroblast-like cell with prominent rough endoplasmic reticulum and a macrophage-like cell with many vacuoles and vesicles (Fig. 2). These cells were similar to to the types of cell seen in rat synovium, and as described by Ghaddially [19]: the type-A phagocytic macrophage-like cell and the type-B secretory fibroblast-like cell, with occasional intermediate cells. Fibroblast-like cells comprised about 90% of the pouch lining layer and macrophage-like cells about 10% by electron microscopy. Collagen fibres were seen in the ground substance of both pouch and synovium and neither had a basement membrane.

The immunohistochemical stains for the macrophage markers muramidase, α-1-antitrypsin and α-1-antichymotrypsin were positive in 30-40% of pouch lining cells, but only in 5% of synoviocytes (Table 1). Histochemical stains revealed that about 40% of the cells in the pouch

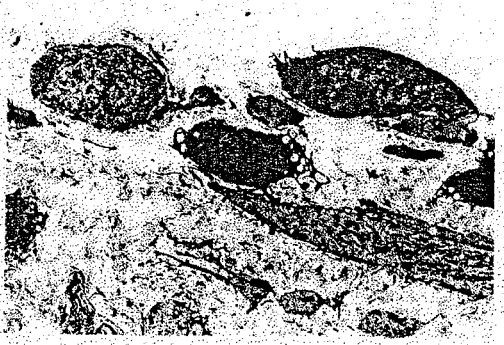

Figure 2
Electron micrograph of unstimulated air pouch lining showing macrophage-like cells (centre and top right) and fibroblast-like cells. X 2550.

lining and synovium were positive for the lysosomal enzymes acid phosphatase and esterase (Table 1). These cells were likely to be macrophages. The cells of the pouch lining and synovium were further characterised by the use of rat monoclonal antibodies to cell membrane antigens (Table 1). Both tissues contained many cells positive for OX-1, identifying them as leucocytes. Cells carrying the Ia antigen, positive for OX-3 and OX-6, were also seen in both tissues, but the pouch lining contained a greater proportion of these mononuclear leucocytes. Such cells were observed in pouch lining cells up to 6 weeks after pouch formation.

The unstimulated air pouch contained insufficient liquid exudate to aspirate, even after opening the pouch. For this reason the minimal fluid exudate adhering to the pouch lining was har-

Table 1
Percentages of cells staining positively for special stains in unstimulated air pouch, synovium and washout.

	pouch	synovium	washout
acid phosphatase	38	42	
esterase	37	36	
muramidase	32	<5	
α-1-antichymotrypsin	45	<5	
α-1-antitrypsin	30	<5	
OX-1 (anti WBC)	60	22	55
OX-3 (anti Ia)	53	21	48
OX-6 (anti Ia)	45	15	28

vested by washing the pouch with 5 ml saline. Washouts of the unstimulated pouch contained few cells (about 200 white blood cells per cubic mm) and consisted of about 80% monocyte/macrophage cells and the remainder variable numbers of lymphocytes, polymorphonuclear nuetrophil and eosinophil cells. Multinucleate giant cells were commonly seen. Many of the cells in the washout carried the Ia antigen (Table 1). Proportions and numbers of cells in the washout hardly varied with time in the unstimulated pouch.

Phagocytic ability

Carbon particles were clearly seen by light and electron microscopy within mononuclear phagocytic cells of the pouch lining 6 hours after India ink injection. Similarly the phagocytic synoviocytes had ingested carbon 4 hours after injection of India ink into the knee joint space.

Acute inflammatory response

The air pouch lining mounted an acute inflammatory response to the locally injected irritants carrageenan, oleyl alcohol, killed *M. tuberculosis* cells and microcrystals characterised at 24 hours by polymorphonuclear and mononuclear phagocytic cell infiltration into the pouch wall and cavity. Histological examination of the inflamed pouch linings showed typical acute inflammatory lesions, with vascular dilation, oedema, margination and extravasation of leucocytes. Fibrin, macrophages and polymorphs covered the internal pouch surface and were present in the washout fluid.

The inflammatory cells in the pouch tissues and exudate after 24 hours were mostly mononuclear for carrageenan type 10 and *M. tuberculosis* cells, mostly polymorphonuclear for urate crystals, and of roughly equal proportions for carrageenan type 7 and oleyl alcohol.

Chronic inflammatory response

Most rats (97%) injected with adjuvant developed a polyarthritis with maximal paw swelling and joint involvement 10-14 days after injection. Synovia taken at 14 days were severely inflamed with proliferation of synoviocytes and infiltration with lymphocytes, macrophages, plasma cells and in some cases polymorphs. Air pouches from the same rats were also inflamed with similar histopathology (Fig. 3). The inflammation was patchy however, with some areas

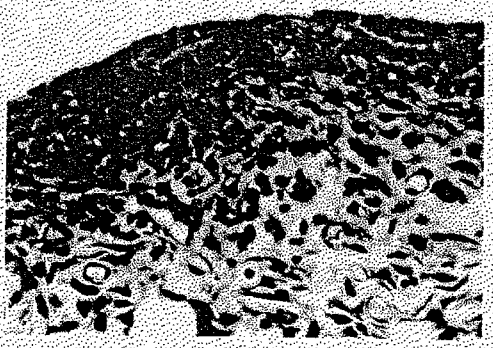

Figure 3
Air pouch tissue 15 days after adjuvant injection into the tail base showing proliferation of the lining cells and infiltration with inflammatory cells and oedema. H + E, X 480.

near normal in appearance and others severely inflamed. Saline washouts of inflamed air pouches from rats with adjuvant disease contained similar cell numbers and types to washouts from unstimulated pouches, suggesting that the inflammation was confined within the pouch tissue.

Samples of synovium and pouch taken during the development of the disease (i.e. less than 10 days after adjuvant injection) showed less severe inflammation.

Connective tissue from a site under the skin of the back remote from the pouch, peritoneal and pleural membranes from rats with adjuvant disease were not inflamed, suggesting that the inflammation in the air pouch lining might not be simply a non-specific phenomenon.

HAGG-induced inflammation

HAGG induced an acute inflammatory response in the air pouch which was characterised by swelling of the pouch lining cells, oedema, margination and extravasation of polymorphonuclear cells (PMN) into the pouch wall and exudation into the cavity with minimal exudate fluid (Fig. 4). The volume of the fluid in the pouch was never more than the injected volume and could only be reliably harvested by saline washout. The leucocyte response to HAGG was dose dependent at 5 hours after injection (Fig. 5) and these cells were 80% PMN. The responses to unaggregated IgG and PBS controls were very low (less than 200; 90% mononuclear cells in 5 ml washout).

HAGG was taken up by the phagocytic cells of the pouch lining within 15 minutes as shown

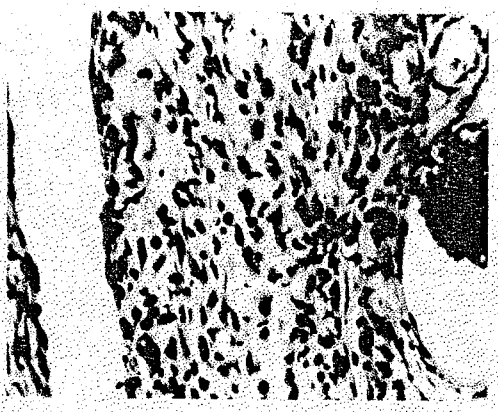

Figure 4
HAGG-induced inflammation of the air pouch showing oedema and inflammatory cell infiltration of the pouch lining 3 hours after injection of 20 mg HAGG into the pouch cavity. A dilated blood vessel is shown on the right packed with polymorphonuclear cells and the pouch cavity is shown as the clear area at left. H + E, X 480.

by using fluorescein labelled antibody to IgG. Further phagocytosis of the aggregates into discrete phagosomes was seen as the inflammation progressed. PMN and macrophages in the exudate also engulfed HAGG.

Vascular leakage was observed from 1.5 hours after HAGG injection. No leakage of fluorescein labelled albumin was seen at 45 minutes or earlier, or in control pouches.

The time course of the acute inflammatory response to HAGG is summarised in Table 2 and Fig. 6. PMN began to appear in the cavity at about 3 hours after HAGG injection, increasing to peak numbers at 12 hours and declining thereafter. In contrast concentrations of the prostaglandin mediator PGE_2 rose sharply to reach a peak at 1.5 to 3 hours and fell to near baseline levels thereafter. No consistent pattern was seen with 6-keto $PGF_{1\alpha}$ or 5-HETE. Concentrations of the lysosomal enzymes β-hexosaminidase, β-glucuronidase and acid phosphatase in the washout followed a similar time course to the leucocytes, peaking at 12 hours. β-glucosidase levels remained low throughout.

Discussion

The air pouch lining is morphologically similar to the synovial membrane. Both tissues contain fibroblast and macrophage-like cells, the apparent proportions depending on the method used to detect them. Immunochemical stains using peroxidase showed 30–50% positive cells in pouch lining and <5–20% positive in synovium. When the enzyme macrophage markers acid phosphatase or esterase were used about 35–40% cells were positively stained in both tissues. Electron microscopy revealed only about 10% cells with clear macrophage morphology in pouch lining. The reason for the difference in apparent macrophage numbers in pouch and synovial membrane characterised by immunochemical stains might be related to differences in antigen expression. Our data confirms and extends and observations of Edwards et al. [1] who found 10-30% Ia positive cells in 14 day old pouches and < 5% in synovium.

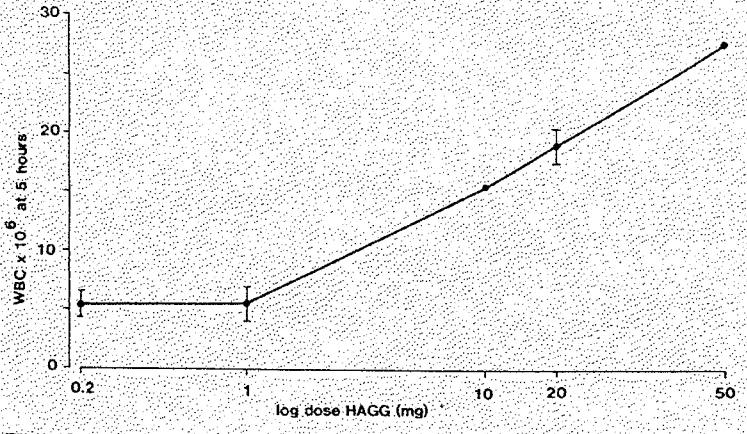

Figure 5
Total leucocyte (WBC) response in 5 ml washout for different doses of HAGG at 5 hours after injection (mean and sem of 4). The counts for PBS and unaggregated IgG were < 200 cells total.

Table 2
Time course of inflammatory response of the 7 day old pouch to HAGG (E) or PBS (C) showing total amount in 5 ml washout of leucocytes (WBC), lysosomal enzymes β-glucuronidase (β-gld), β-hexosaminidase (β-hex), acid phosphatase (AcP), β-glucosidase (β-glu), prostanoids PGE_2 and 6-keto $PGF_{1\alpha}$, and the lipoxygenase pathway product 5-HETE. (Not detectable = ND)

hours post injection		WBC	β-gld	β-hex	AcP	β-glu	PGE_2	6-keto $PGF_{1\alpha}$	5-HETE	
		× 10^6	nmol/min				← ng →			
0 (pre)			0.22	0.26	0.28	ND	0.63	3.30	1.62	25.50
1.5	E		0.62	0.89	1.83	1.05	0.80	7.92	15.30	9.82
	C		0.20	—	—	—	—	4.35	9.35	21.00
3	E		4.08	1.85	3.90	2.20	0.85	7.58	9.56	8.05
	C		0.33	0.47	0.17	ND	0.60	7.50	8.50	11.00
6	E		21.00	1.10	3.42	2.65	0.95	3.33	1.90	5.25
	C		3.30	0.77	1.58	0.95	4.35	1.45	1.70	6.75
12	E		36.08	2.05	5.09	4.50	1.15	3.48	1.42	19.37
	C		0.37	—	—	—	—	1.95	3.25	5.00
24	E		12.10	0.59	1.84	1.20	1.30	2.00	1.33	4.50
	C		3.92	—	—	—	—	2.45	1.40	6.75

We have shown that the rat air pouch is also functionally similar to synovium. It can phagocytose particles and is able to mount an acute inflammatory response to a variety of irritants, with an influx of inflammatory cells and minimal exudate.

The rat air pouch develops a chronic inflammation in response to systemic adjuvant injection concurrently with the synovitis, both becoming most severe 10-14 days after injection. The pouch inflammation was unlikely to be merely a consequence of non-specific connective tissue inflammation because no inflammatory cells were found in peritoneal or pleural membranes or other connective tissue. These observations show another similarity between pouch lining and synovium. In contrast to adjuvant synovitis the pouch inflammation was rather patchy and confined to the tissues with no inflammatory cells in the exudate, suggesting a largely proliferative effect of the adjuvant in pouch lining tissue.

There are major differences between the tissues which indicate that the pouch lining could also be regarded validly as simply the expected result of new cavity formation in connective tissue. In particular the pouch lining is much thicker

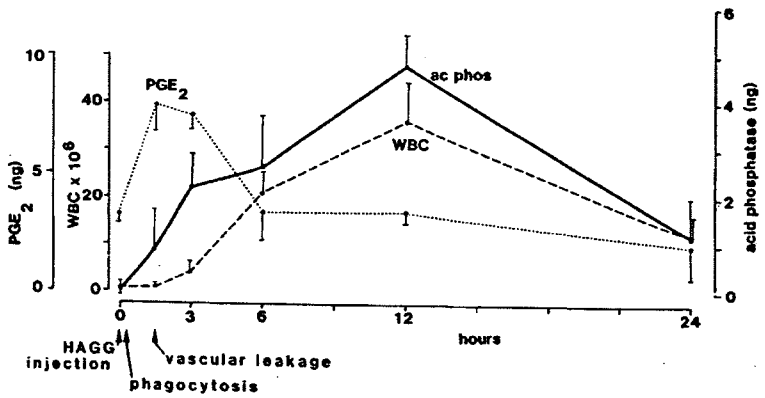

Figure 6
Summary of the time course of HAGG-induced inflammation. HAGG was observed phagocytosed by the pouch lining cells 15 mins after injection and vascular permeability to fluorescein-labelled IgG was observed to increase 1.5 hours after the induction of inflammation. The graphs show that PGE_2 concentrations peaked at 1.5-3 hours, dissociated from the later peak of polymorphonuclear leucocytes and lysosomal enzymes. The total PGE_2, acid phosphatase and cell count (WBC) in 5 ml washout are shown (mean and sem of 4).

than synovium. We have been unable to detect any hyaluronate in the pouch lining or washout by electrophoretic or histochemical means (unpublished observations), unlike synovium which actively secretes hyaluronic acid. This may be one reason for the minimal fluid exudate formed in our inflamed air pouches. Alternatively the physical barrier of the pouch lining-air interface may impede the passage of cells and fluid into the cavity.

In our hands the inflamed 7 day air pouch did not yield sufficient exudate to harvest reliably and so we used saline washouts to recover cells and soluble mediators. Other workers claim to have measured exudate volume in acutely inflamed air pouches [7-8]. The presence of fluid exudate in those studies may be due to differences in irritants or strains of rats, or possible endotoxin contamination.

Despite these differences we believe that the 7 day old air pouch provides a readily accessible *in vivo* culture chamber in which to study inflammation, illustrated by our use of the model to study the acute response to HAGG (model IC) and its time course by washout of cells and mediators.

The response to HAGG was typical of an acute inflammatory response with PMN infiltration of the pouch wall and cavity in a dose dependent manner. Maximal numbers of polymorphs and concentrations of the lysosomal enzymes β-glucuronidase, β-hexosaminidase and acid phosphatase were observed temporally dissociated from the earlier peak of PGE_2. A similar dissociation between cellular infiltration and prostaglandin concentrations was seen in monosodium urate crystal induced inflammation in the pouch [10]. The results suggest that the infiltrating PMN were the source of the enzymes but not the prostaglandins. Polymorphs were unlikely to be the source of prostanoids as they metabolise arachidonic acid mainly by the lipoxygenase pathway to produce leukotrienes. The lack of consistent pattern of 5-HETE production may relate to problems with the assay method or to the relatively low cell counts. We observed phagocytosis of HAGG by pouch lining cells as early as 15 minutes after injection, and increased vascular permeability at 1.5 hours. We postulate that rapid uptake of HAGG by pouch lining cells results in the release of vasoactive substances which increase vascular permeability and PGE_2 synthesis. PMN infiltration, phagocytosis of HAGG and degranulation resulting in lysosomal enzyme release follow. Similar mechanisms may apply in the initiation of IC-mediated synovitis which is implicated in RA. Pharmacological agents could be used to elucidate the mechanisms of the inflammatory process.

We do not consider the air pouch, as we describe it, to be a suitable model of chronic synovial inflammation for the following reasons. The reactivity of the air pouch varies with age [20-21]. An important difference between the air pouch and the synovial joint is the absence of cartilage in the pouch. It is known that chronic joint inflammation may occur as a result of frustrated phagocytosis of IC bound to the cartilage collagen [14]. In pouch tissue IC are taken up by the pouch lining cells, producing an acute inflammation, but are then rapidly cleared. We have been unable to demonstrate trapping of IC by the pouch lining cells beyond the acute inflammatory stage. Other important differences include the lack of movement and any associated trauma, and the absence of synovial fluid in the pouch.

The 6-7 day old air pouch superficially resembles synovium and it has been suggested that such air pouches could be used to model the chronic synovial joint inflammation which is a feature of rheumatoid arthritis. Chronic granulomas have been produced by injecting potent irritants into newly formed, i.e. 24 hours old, air pouches [2-5, 22-23]. Interesting general information about mechanisms of chronic inflammation can be obtained using such pouches but they bear little resemblance to the synovial cavity.

Chronic antigen-induced inflammation has been produced in 24 hour old pouches using modified BSA [24] or repeated challenge with BSA [25] in carboxymethyl-cellulose vehicle. In these studies the unsensitised animals also showed an acute inflammatory response. Recently Edwards et al. [26] demonstrated a chronic antigen-induced inflammation in response to challenge with pertussis vaccine in sensitised animals. In all these studies of chronic antigen-induced inflammation the animals were sensitised with antigen and Freund's complete adjuvant. The chronic inflammation observed may have been partly due to the pouch tissue response to adjuvant which we describe. Interesting work has been published recently on destruction of cartilage implants into inflamed air pouches [27-28].

We believe that the 7 day old air pouch is best suited for the study of acute inflammatory reponses as illustrated by our work with HAGG. The differences between the air pouch and the synovial joint highlight the need for caution in extrapolating from inflammation induced in the pouch model to joint synovitis.

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