Quantification of the host response to implanted polymers *in vivo* by flow cytometry

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A series of medical grade polymers and one non-medical grade polymer, in the form of tubes closed at one end, were implanted subcutaneously into the dorso-lumbar region of rats. The samples were retrieved after 1, 2, 5, 7 and 14 days and the exudate which formed within the lumen of the tubes analysed by flow cytometry. Positivity for each of six antibodies: CD45RO (pan leucocyte), OX42 (macrophage/monocyte), CD5 (T-lymphocyte), CD45RA (B-lymphocyte), Interleukin-2 (IL-2) receptor and Major Histocompatibility class II (MHC-II) antigen were tested. The volumes of exudates and their total cellularities varied greatly from material to material, but there was no relationship between the two. Silicone, for example, had the greatest exudate volume at all time periods, but the lowest cellularities after day 5. Large numbers of granulocytes and macrophages were observed in all materials, with smaller populations of T-lymphocytes, which also demonstrated high IL-2 receptor expression. High levels of MHC-II expression were observed and attributed mainly to the macrophage population. All materials demonstrated the very reactive nature of this type of model: a massive inflammatory reaction continuing right up to 14 days in all cases, seemingly acute in cellular composition with high immunological activity. It is concluded that flow cytometry is an extremely useful tool in probing the interaction of cells with artificial materials.

1. Introduction

The host response to an implanted biomaterial is now quantified quite reproducibly when it is possible to explant the implanted material with the surrounding tissue [1], using a combination of immunohistochemistry with computer image analysis. The different cell types are identified in the tissue using highly specific monoclonal antibodies, which routinely mark inflammatory cells like macrophages, T-lymphocytes, B-lymphocytes and granulocytes. Image analysis then returns information about cell numbers and their position relative to the implanted material.

The analysis of cell exudates cannot be approached in this manner and has traditionally been assessed using human judgement to estimate the size and type of cell response. In this paper we have attempted to show that the analysis of a response in environments where there is no direct implant fixation into an organ or tissue can be reproducibly performed by more modern techniques, namely flow cytometry. Less operator judgement is required by utilizing the same antibody technology and allowing information to be returned not only on cell numbers, but specific cell receptor expression within subsets of cells. Techniques like this are becoming increasingly important as materials are being designed to produce a specific response within a body. Our ability to assess these subtle differences is of great importance in further enhancing our ability to predict and control both material and host responses.

Not only can this technique be used to quantify the types of cells present, but also their activation state. Knowledge of the manner in which inflammatory cells are activated is critical to the understanding of their interaction with biomaterial surfaces. The data presented here represents a preliminary study to determine the efficacy of flow cytometry in assessing materials and implantation sites that cannot be assessed using other techniques.

2. Materials and methods
2.1. Materials
With the exception of red rubber latex, the biomaterials used represent a range of medical grade polymers which are commonly used in surgical procedures. Silicone was standard grade with a shore hardness of 50 from Altec, Alton, Hampshire, UK. PVC was standard grade with a shore hardness of 80 from Portex, Hythe. Kent, UK. PTFE was from Altec, Alton, Hampshire UK. Pellethane, a commonly used aromatic polyetherurethane, was 2363/90A grade from Dow, Midland, Minnesota, USA and extruded by Viggo-Spectramed, Swindon, Wiltshire, UK. Red rubber latex was standard laboratory grade from BDH/Merck, Lutterworth, Leicestershire, UK. All had an internal diameter of 6 mm. They were cut into 20 mm-long lengths (representing an internal volume of 565 µl) and sealed at one end with medical grade

silastic adhesive. Cleaning and sterilization were performed by washing in distilled water in an ultrasonic water bath for 2 h then autoclaving.

2.2. Implantation

Samples were implanted subcutaneously into the back of six-month-old black and white hooded Lister rats of the Liverpool strain, all weighing in the range 300–310 g. A single rat was used for each material per time period, with two samples implanted into each, one either side of the spine, lying on top of the dorso-lumbar muscle. The rats were sacrificed after 1, 2, 5, 7 and 14 days. The implants were carefully retrieved and the exudate which had collected in the lumen of the tubes during the period of implantation harvested by centrifugation.

2.3. Flow cytometry

Exudate cellularity was assessed by counting the number of particles with a diameter greater than approximately 5 μm. Each exudate was diluted with a buffered and filtered (to 0.22 μm) saline solution so as to have equal cell concentrations of approximately 5 × 10⁶ cells/ml. 20 μl diluted exudate were incubated with 10 μl of each of six different monoclonal antibodies, all directly conjugated with fluorescein isothiocyanate (FITC). Mouse monoclonal antibodies specific for rat antigens from Serotec (Oxford, U.K.) were used: CD45RO (M.ab MRC-OX1), macrophages (M.ab MRC-OX42), CD5 (M.ab MRC-OX19), IL-2 receptor (M.ab MRC-OC39) and MHC-II antigen (M.ab B F-1). The B-lymphocyte marker CD45RA (M.ab MRC-OX33) was bought from Sera-Lab (Crawley Down, Sussex, UK). These were incubated for 30 min at 4°C in the dark. Samples were diluted with 500 μl filtered saline (FACSFlow, Becton Dickinson, Oxford, UK) prior to analysis. Flow cytometry was performed using a Becton Dickinson FACSort (San José, California, USA). Relative fluorescence was measured for all samples and positivity tested by reference to a negative control. This was provided by incubating six tubes of red blood cells diluted with saline to 5 × 10⁶ cells/ml with each antibody as before (red blood cells have none of the appropraite antigens). All incubations were performed at 4 °C to limit the degree of non-specific binding. The antibody concentration which gave the greatest separation of negative and positive peaks in a mixture of negative and positive cell types (at a cell concentration of 5 × 10⁶ cells/ml) was used. 30 000 cells were counted for each exudate/antibody combination and cells with a diameter of less than approximately 5 μm were thresholded-out electronically.

3. Results

The data in Figs 2–7 represent the total numbers of cells present in the exudates, rather than their concentrations. The volume of exudate was not correlated to the numbers of cells present, and since fluid production is therefore independent of cell production, pre-

senting the data as cell concentrations was thought to be irrelevant and confusing.

Silicone had the greatest volume of exudate for all time periods after day 1 (Fig. 1), with a reduction in exudate volume at 14 days. However, the cellularities of these exudates were not the greatest (Fig. 2), and did not follow the trend in silicone's increasing volume of exudate with time. The stimulation of cells into this material seemed to be slow (2 days) but once started there was a constant influx of granulocytes and macrophages into the sample with a low number of activated T-lymphocytes (Fig. 3). Large numbers of the macrophages were in an activated state (MHC-II positive), with MHC-II expression lagging behind macrophage appearance in the exudate.

Latex had consistently the lowest exudate volumes after the 1 day time period (Fig. 1), and some of the highest cellularities (Fig. 2). There was early stimulation of inflammatory cells into these samples (Fig. 4), then a tailing off of the cellular response by 2 and 5 days, before the cellularities of macrophages and granulocytes rose again. This latter rise may have been stimulated by an increase in T-lymphocyte number at 5 days: all of the T-lymphocytes observed at all time
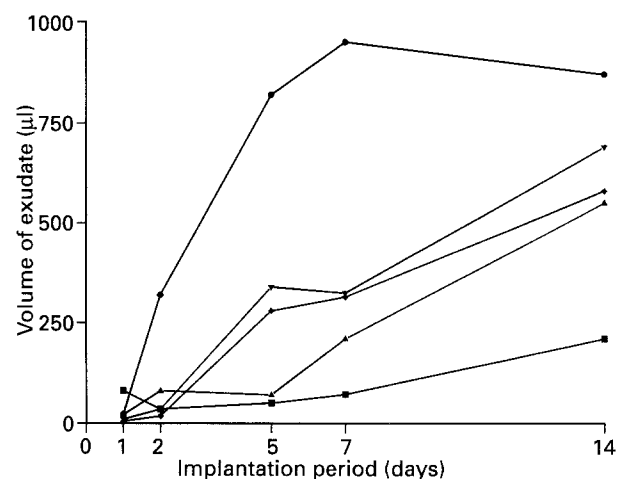

Figure 1 Volumes of exudate retrieved from implanted materials (● silicone; ■ latex; ▲ PVC; ▼ PTFE; ◆ pellethane).
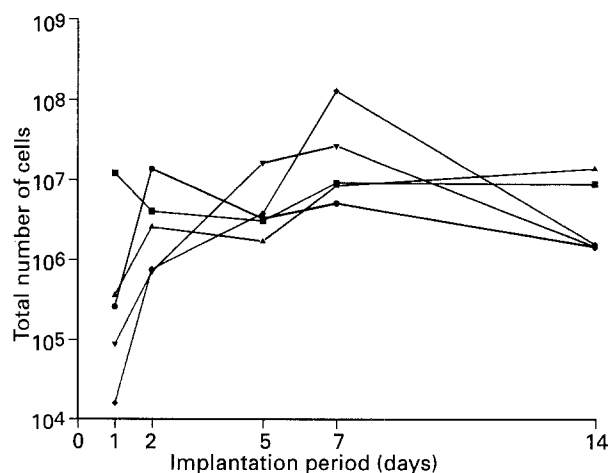

Figure 2 Total cellularities of exudates from implanted materials (● silicone; ■ latex; ▲ PVC; ▼ PTFE; ◆ pellethane).
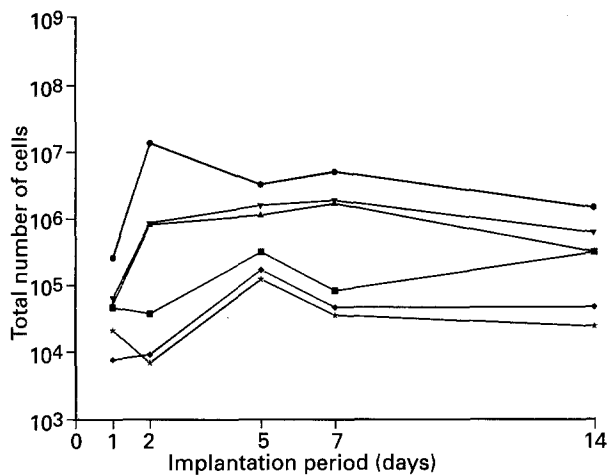
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Figure 3 Composition of exudate retrieved from implanted silicone samples (● total; ▲ granulocyte; ▼ macrophage; ◆ CD5 positive; ✳ IL-2 positive; ■ MHC-II positive).
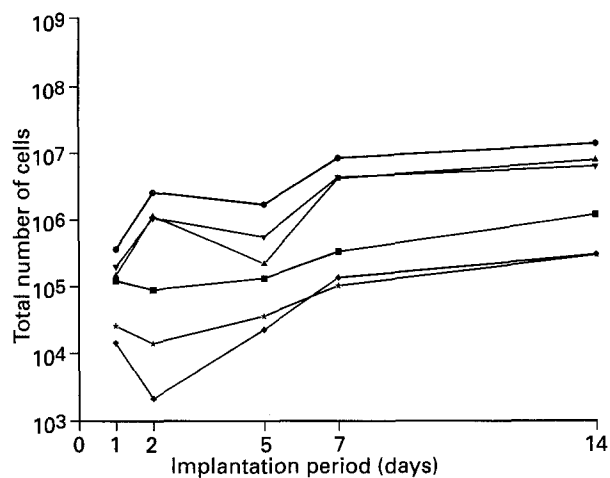

Figure 5 Composition of exudate retrieved from implanted PVC samples (● total; ▲ granulocyte; ▼ macrophage; ◆ CD5 positive; ✳ IL-2 positive; ■ MHC-II positive).
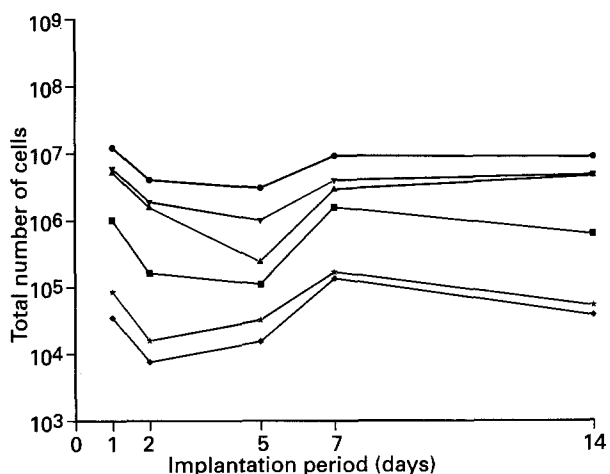

Figure 4 Composition of exudate retrieved from implanted rubber latex samples (● total; ▲ granulocyte; ▼ macrophage; ◆ CD5 positive; ✳ IL-2 positive; ■ MHC-II positive).
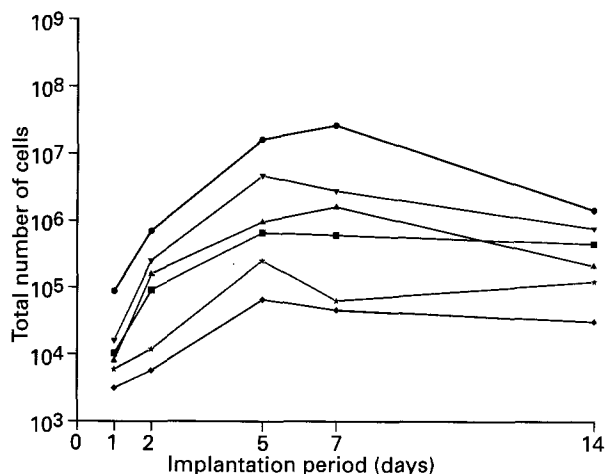

Figure 6 Composition of exudate retrieved from implanted PTFE samples (● total; ▲ granulocyte; ▼ macrophage; ◆ CD5 positive; ✳ IL-2 positive; ■ MHC-II positive).

periods were positive for the IL-2 receptor. Although the T-lymphocyte response is very small compared to granulocytes and macrophages, their rôle in a sustained acute inflammatory response should not be underestimated, particularly given their state of activation (IL-2 receptor expression). A large proportion of the macrophage population also showed activation, the MHC-II positive graph mirroring the macrophage graph but smaller in magnitude. However, in this case, MHC-II expression did not lag macrophage number.

The exudate volume from PVC did not rise consistently with time, showing an early peak at 2 days, then a small drop at 5 days with a steady rise after that period (Fig. 1). The total cellularity reflects this, as does the individual granulocyte and macrophage numbers. MHC-II expression on the macrophage population showed a steady increase with time, following IL-2 receptor expression more closely than it did to macrophage number. The T-lymphocyte population did not follow a steady increase (Fig. 5). This showed an early peak at 1 day, dropping to a minimum at day 2, at which point the granulocyte and

macrophage numbers were rapidly increasing. The T-lymphocyte numbers steadily increased to 5, 7 and 14 days, perhaps again implicating them in the continued stimulation of inflammation in these materials. However, there was some variation between CD5 and IL-2 receptor expression at 2 days.

The volume of exudate from PTFE tubes rose fairly consistently with time with a slight drop at 5 days (Fig. 1). The cellularity, however, showed maximum numbers of cells at the 5-day and 7-day time periods. Macrophage numbers reached a peak at 5 days while granulocytes reached a maximum at 7 days (Fig. 6), with both cell types tailing off at 14 days, the granulocyte numbers having the greatest decrease. T-lymphocytes peaked at 5 days, but did not seem to have the same relationship to macrophage or granulocyte numbers as was the case for PVC and latex. Once again, IL-2 receptor expression in the T-lymphocyte population accounted for more cells than CD5, at all time periods. MHC-II expression closely followed the macrophage number as in the case of latex, with a gradually increasing proportion of the macrophages expressing the MHC-II antigen.
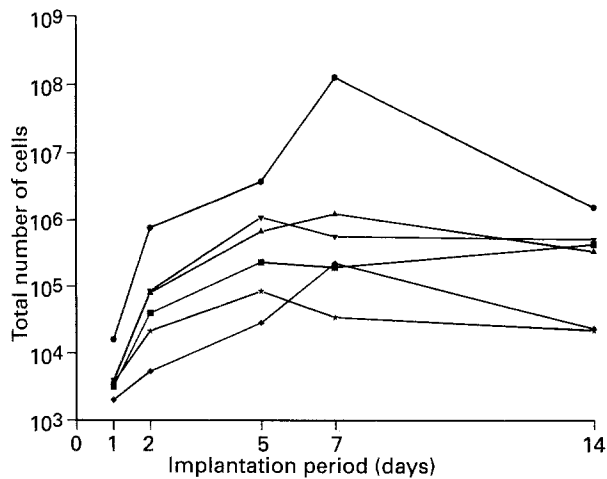
Figure 7 Composition of exudate retrieved from implanted Pelle-thane samples (● total; ▲ granulocyte; ▼ macrophage; ◆ CD5 positive; ✳ IL-2 positive; ■ MHC-II positive).

The Pellethane exudate volume followed that of PVC very closely (Fig. 1), but there were few similarities in the cellular response by the 14-day time period. Pellethane showed a steady influx of cells, similar to that observed with PTFE at the 1, 2, 5 and 7 day time periods, with the same reduction at 14 days (Fig. 7). However, the T-lymphocyte response in the Pelle-thane exudates was very much greater at 7 days (in fact the largest at 2.24×10^5), with no reflection on granulocyte or macrophage numbers a week later at 14 days. This may be due to the lack of IL-2 receptor expression in the T-lymphocyte population at 7 days, perhaps indicating that this population is predominantly comprised of maturing cells and the acute phase of inflammation is resolving. The activation state of the macrophages is equally unaffected by the fluctuation in antigen expression in the T-lymphocyte population, with the proportion of activated macrophages increasing after day 2 until almost 100% of the population expressed the MHC-II antigen at the 14-day time period.

No B-lymphocyte expression was observed in any of the exudates and was therefore not included on any of the graphs.

As may be gleaned from Figs 3-7, the total cell numbers are not totally accounted for by macro-phages, granulocytes, T- or B-lymphocyte populations. This discrepancy varied from virtually nothing to 98% (Pellethane, 7 day) of the total cell number. A number of the exudates had visible evidence of red blood cell contamination, which is probably due to in-bleeding during the explanation procedure. The use of specific antibody technology ensured that the presence of red blood cells did not influence the counts of other cell types.

4. Discussion

The subcutaneous model was selected for this study because, although it is basically an "acellular" environment at the start, once a material is implanted, there is exudate formation brought about in two ways: (i) by the physical presence of the implant; and (ii) by

the chemical composition of the implant. This model is not ideal for "real" tissue responses since there is a lack of structure and real tissue resolution, other than granulation and isolation of the implanted material. In addition, the inability to anchor the implanted device without suturing to other tissues can be a complicating factor. As an *in vivo* model that causes minimal tissue damage on material implantation, it is ideal for examining the potential of a material to stimulate or regulate an inflammatory reaction in the presence of large numbers of inflammatory cells and extracellular "factors". This is provided that the assessment procedure does not involve an analysis of the inflammatory reaction within the surrounding tissue but solely components within the exudate.

This preliminary study was performed to test the ability of this animal model, in combination with flow cytometric analysis of the resultant exudate, to compare the cellular responses to implanted materials, If successful this model would provide a means to examine certain types of material that do not lend themselves to analysis in other implantation models.

Massive numbers of inflammatory cells were detected in the exudates retrieved from the five types of material. Some of the differences in the cell numbers between materials were very large. The number of cells observed in the exudates was not related to the volume of exudate produced. Comparing Fig. 1 with Fig. 2 clearly demonstrates this.

The granulocyte and macrophage populations follow each other very closely at all time periods for all samples. There are slight dips in the granulocyte number at 5 days in the latex and PVC samples, but on the whole the response between the two cell types was identical. This is somewhat surprising in that the granulocyte response was sustained through to the later time periods and the macrophage response began at the very early time periods. There was obviously potent stimulation of cellular migration for these cell types in this animal model. The sustained high cell numbers coupled with a constant presence of T-lymphocytes indicates a continued stimulus by all materials to maintain what was a massive inflammatory response. Future investigations will extend the implantation time period to determine for how long this response continues, and will also involve a more extensive panel of antibodies to determine more specifically the activation state of the macrophages and the subsets of T-lymphocytes. In conjunction with CD5 and IL-2 receptor antibodies, CD4 and CD8 expression should be analysed to determine this T-cell response and therefore its significance.

CD5 and IL-2 receptor positivity for T-lympho-cytes shows overlap in many samples, suggesting that T-lymphocytes expressing IL-2 receptors sometimes outnumber CD5-positive lymphocytes, CD5 supposedly representing a definitive T-lymphocyte marker. This is not totally unexpected since CDS expression is linked to T-lymphocyte activation. CD5 expression also changes as T-lymphocytes mature and differs within subsets of T-cells [2]. What is significant is that, in all samples, the numbers of cells expressing these tow antigens follow the same pattern, demonstrating a

high degree of sensitivity and accuracy in this technique.

MHC-II expression has been presented in the results as relating to the macrophage population in the exudate, and to be a reflection of their state of activation. Considering the low numbers of T-lymphocytes, the lack of B-lymphocytes and that the major percentage of MHC-II expressing cells possessed forward light and 90° light scatter characteristic of macrophages, then this is a reasonable assumption. However, there is some MHC-II expression in other regions of the forward and 90° light scatter plot, particularly where we observed lymphocytes. It is not uncommon for many cell types to express MHC-II antigens other than macrophages: B-lymphocytes, T-lymphocytes, endothelial cells and smooth muscle cells máy also express MHC-II antigens [3]. High MHC-II expression in an area could be indicative of an immunologically activated site, MHC-II molecules being required for antigen recognition by T-helper cells and therefore initiation of an immune response. Abundant expression is also observed in autoimmune diseases as well as chronic inflammatory reactions, may be indicating a loss of immunological control in an area of inflammation.

5. Conclusions
From our data it would appear that all the materials assessed stimulated an inflammatory response, which is acute in its cellular composition, but far from over by 14 days. There is considerable IL-2 expression on the T-lymphocytes and thigh MHC-II expression, with massive numbers of granulocytes accompanying the large numbers of macrophages.

There are differences in the amount of exudate produced in response to each material, and also massive differences in the cellular response to different materials. This study only demonstrates the potential of this kind of technique at this stage, and hints at our ability to analyse a materials effect on the body in the future.

References
1. J. A. HUNT, D. G. VINCE and D. F. WILLIAMS, *J. Biomed. Engng* 15 (1993) 39.
2. C. REITER in "Leucocyte typing IV: white cell differentiation Antigens" (Oxford University Press, Oxford, 1989) p. 331.
3. A. L. RAMSHAW and D. V. PARUMS, *Histopathology* 17 (1990) 543.