Analysis of the inflammatory exudate surrounding implanted polymers using flow cytometry

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Cellular responses to silicone and latex were investigated using flow cytometry, to determine the cells involved in the inflammatory responses and to characterize the differences in the response between these materials, if any. A panel of 11 monoclonal antibodies were selected to cover the range of cells that could be involved in the response, each antibody being directly conjugated with fluorescein isothiocyanate (FITC). The materials were implanted subcutaneously into rats in tubular form; the tubes were sealed at one end with Silastic adhesive. Two tubes per animal were implanted, using four animals per time period for 1, 2, 5 and 7 day implantation periods. After these times the animals were sacrificed and the tubes retrieved and then spun to harvest the exudate from the lumen. This exudate was analysed using flow cytometry. Significant and reproducible differences in cell number and antibody positivity were observed between these two materials. Latex had a much larger cellular response and showed significant increases in antibody positivity that involved macrophages or granulocytes of unusual size and granularity. Overlap between antibody positivity made specific characterization difficult and led to many questions about the effect of exposure to a material and its effect on cell morphology and phenotype, particularly in the case of macrophages.

1. Introduction

Previously we have investigated the ability of flow cytometry to evaluate inflammatory responses in tissue exudates. Flow cytometry was demonstrated to have potential in determining the cellular components in acute inflammatory responses or in inflammation involving the production of an inflammatory exudate [1]. This study investigates further the inflammatory exudate that may be involved in the body's response to an implanted material by studying the difference in response to two different polymer materials, rubber latex and silicone, implanted subcutaneously in tubular form into the backs of black and white hooded Lister rats of the Liverpool strain. Of particular interest was the short-term inflammatory response to these materials, the types of cells present and their number. Inflammatory exudates were analysed using flow cytometry to calculate total cell numbers and the proportions of macrophages, lymphocytes and granulocytes present by analysis of their forward scatter (volume) against side scatter (granularity) characteristics, in conjunction with specific monoclonal antibody cell markers to detect and analyse subsets of cells, particularly macrophages, for their antibody positivity and their change in positivity over time and with varying implanted material.

There are many subtle effects involved in inflammation and an ever-increasing number of important interactions involving cells, proteins, materials and cytokines to consider when investigating a body's response to an implanted material. The permutations for complexity increase with each additional cell or cytokine detected and investigated. Ideally we would like to isolate the drivers of a response and control these regulators in a manner of our choosing to harmonize the material and implant interaction. This is a clear and obviously desirable objective, but successes to date have lead to increasing complexity in the response and further complicate the analysis of interactions by involving more detailed investigations. Is such a complicated response really going to depend on single key factors? It is known that this well-developed defence mechanism has many feedback loops with independent factors able to exert both positive and negative effects. Many studies have proved the effects on isolated areas of the overall response *in vitro*, and many cells and protein signals have been observed and measured *in vivo*. Changes in cellular responses can be seen by changing the implanted material [2–4] and also by changing the period of implantation [5, 6].

Complicated though the response is to implanted materials, it is possible to trick or mimic the body once some of the complexities are understood, and it is here that these seemingly over-developed investigative tools become important in their ability to analyse

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subtle changes in a body's response. In order to study the real effects of altering a signal, the analysis must be very specific, precise and reproducible.

2. Materials and methods

2.1. Materials

The silicone used was standard grade with a shore hardness of 50 obtained from Altec (Alton, Hampshire, UK). The red rubber latex used was standard laboratory grade from BDH/Merck (Lutterworth, Leicestershire, UK). Both materials were supplied as tubing, and had an internal diameter of 6 mm. They were cut into 20 mm lengths and sealed at one end with medical grade Silastic adhesive (Dow Corning, UK). Cleaning and sterilizing were performed by washing the materials in distilled water in an ultrasonic water bath for 2 h, then autoclaving.

2.2. Implantation

Samples were implanted subcutaneously into the back of 6 month old black and white hooded Lister rats of the Liverpool strain, all weighing in the range 300–310 g. Four rats per time period were used for each material, with two samples implanted via blunt disection into each animal, one sample either side of the spine, lying on top of the dorsolumbar muscle. The rats were sacrificed after 1, 2, 5 or 7 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. The exudates were diluted with a buffered and filtered (to 0.22 μm) saline solution, to provide equal cell concentrations of approximately 5×10^6 cells ml^{-1}.

2.3. Flow cytometry

A 20 μl sample of diluted exudate or culture was incubated with 10 μl of each of 11 different monoclonal antibodies, all directly conjugated with FITC, to determine specific cell types. The following mouse monoclonal antibodies specific for rat antigens were obtained from Serotec (Oxford, UK) : CD45RO (clone no. MRC-OX1) (a marker for leukocyte common antigen), CD11b/c (clone no. MRC-OX42), ED2 (clone no. ED2) (a marker for mature macrophages), CD14 (clone no. ED9) (a marker for monocytes, macrophages and granulocytes), α/β (clone no. R73), CD5 (clone no. MRC-OX19) (a marker for T-lymphocytes), interleukin-2 (IL-2) receptor (clone no. MRC-OC39) (a marker for activated T-lymphocytes) and granulocytes (clone no. HIS48 and clone no. MOM/3F12/F2). The B-lymphocyte marker CD45RA (clone no. MRC-OX33) was obtained from Sera-Lab (Crawley Down, Sussex, UK). MCP-1 antibody (a maker for monocyte chemotactic protein 1). Red blood cells in the exudate samples were lysed prior to flow cytometry, using 300 μl fluorescent activated cell sorter (FACS) lysing solution (Becton Dickinson, San José, California, USA) at the recommended concentration added to the stained cell suspensions (30 μl) and incubated at room temperature in the dark for 12 min. The lysing action was stopped by the addition of 2.2 ml filtered saline (FACSFlow, Becton Dickinson, Oxford, UK). Non-lysed samples were diluted with 300 μl FACSFlow prior to analysis. Flow cytometry was performed using a Becton Dickinson FACSort (San José, California, USA).

Volume and granularity distributions were obtained by comparing cell forward light scatter with 90° light scatter. Relative fluorescence was measured for all samples, and positivity tested by reference to a negative control. This was provided by incubating both cultured cells and exudates with an antibody of the same subclass as the test antibodies and also directly conjugated with FITC. All incubations were performed in the dark and at 4 °C to limit the degree of non-specific binding. The minimum antibody concentration which gave the highest fluorescence reading in a tube of positive cell types (at a cell concentration of 5×10^6 cells ml^{-1}) was used. A total of 30 000 cells were counted for each exudate or culture/antibody combination, and cells with a diameter of less than approximately 5 μm were thresholded-out electronically.

2.4. Statistical analysis

Statistical significance in the data for each antibody was tested by multi-variate analysis of variance (MANOVA) using a Duncan multiple range test cross checked with a Waller Duncan *k*-ratio *t* test performed on all main effect means (region means for each antibody in each material at both time points). These tests compared the effects of the two materials across the 5 and 7 day time periods. The program used was SAS version 6.04 (SAS Institute, USA).

3. Results

The volume of exudate and the number of cells in both materials remained low for the 1 and 2 day time periods, then increased at 5 and 7 days. Latex had greater numbers of cells at all time periods except 1 day. Positivity for the 11 antibodies demonstrated the difference in the responses to these two types of material. At the 1 and 2 day time points, insufficient exudate was retrieved to allow antibody analysis and flow cytometry, therefore only data for the 5 and 7 day time points is reported. Plots of volume (forward scatter) against granularity (side scatter) (Figs 1 and 2) show the six regions the data was divided into (R1–R3 and R5–R7), determined by the density of clusters in the two materials; Table I shows the large differences in the numbers of cells observed between silicone and latex in these regions.

Latex stimulates a significantly larger macrophage and granulocyte CD14 positive population at day 5 (Fig. 3), but by day 7 the relative percentage of positive cells was the same. At day 5, R6, R7 and R1 with latex had significantly more CD14 positive cells than silicone. By day 7 only R2 CD14 positive cells were significantly greater ($P < 0.05$) (Fig. 3). With silicone the positivity in each region remained the same; with latex the positivity fell in every region with time

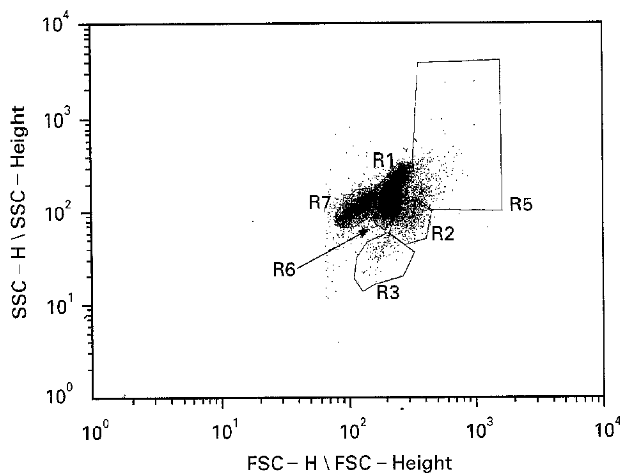

Figure 1 Scatter plot for latex 7 day sample.

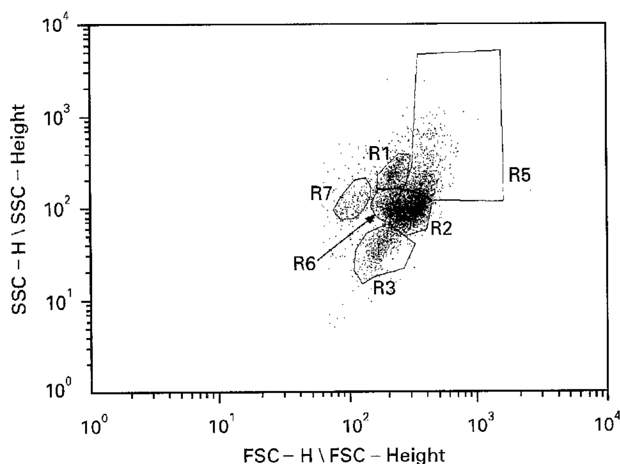

Figure 2 Scatter plot for silicone 7 day sample.

TABLE I Total cell number ($\times 10^3$/ml)

Region	Silicone Day 5	Silicone Day 7	Latex Day 5	Latex Day 7
Total	585.4	1824	3250	15 820
R1	20.87	107	271.9	2288
R2	269.9	794.6	797.7	818.7
R3	58.56	294.8	33.0	87.0
R5	64.9	263.8	233.8	361.5
R6	20.2	59.17	759.3	3193
R7	34.4	48.6	496.5	5582

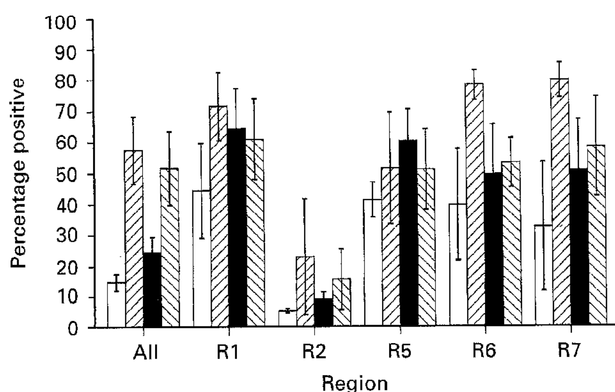

Figure 3 CD14 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

(although the number may not have). In R6, the latex 5 day percentage positivity was significantly greater ($P < 0.05$) than silicone 5 or 7 day and latex 7 day.

MCP-1 positive cells (monocytes, macrophages and polymorphonuclear leukocytes) followed a similar pattern. For R6, latex 5 and 7 day values were significantly greater ($P < 0.05$) than silicone 5 and 7 day values, with no significant difference between 5 and 7 day the data within the latex or silicone groups separately (Fig. 4).

There were no significant differences between silicone and latex in positivity for the IL-2 receptor at day 5 or 7 (Fig. 5). ED2 showed a significant difference between the materials, latex having more positivity. In R2, latex 5 and 7 day samples were significantly more positive ($P < 0.05$) than silicone 5 and 7 day samples. Latex samples were more positive than silicone samples, but with no significant difference between 5 and 7 day the data within the latex or silicone groups separately (Fig. 6).

CD45 showed high percentage positivity in all regions for both materials at 5 and 7 days, with no significant difference between the percentages (Fig. 7). With CD11b/c, latex 5 and 7 day samples were significantly more positive than silicone 5 and 7 day samples in all regions. There were no significant differences between the 5 and 7 day samples of each material except in R1, where silicone 5 day positivity was significantly greater than silicone 7 day positivity (Fig. 8).

In R3, α/β positive cells were significantly greater in silicone 5 and 7 day samples than in latex 5 and 7 day samples (Fig. 9). For CD5 positivity there were no

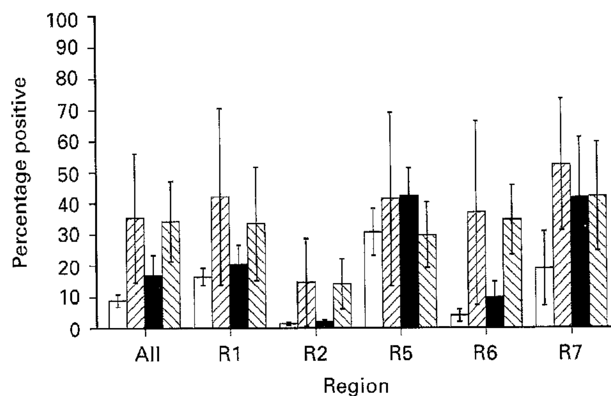

Figure 4 MCP-1 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

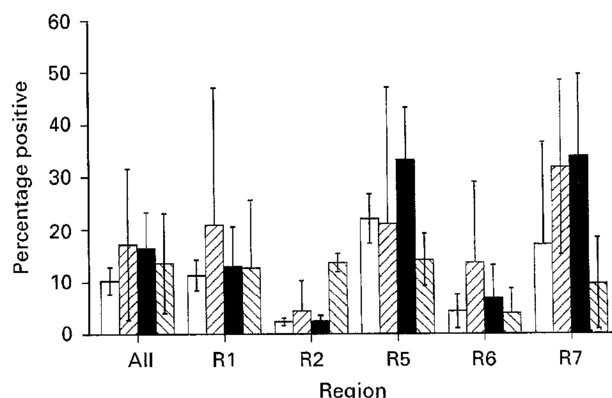

Figure 5 IL-2 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

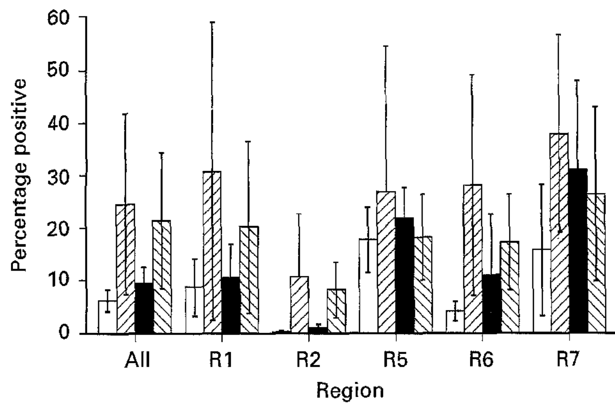

Figure 6 ED-2 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

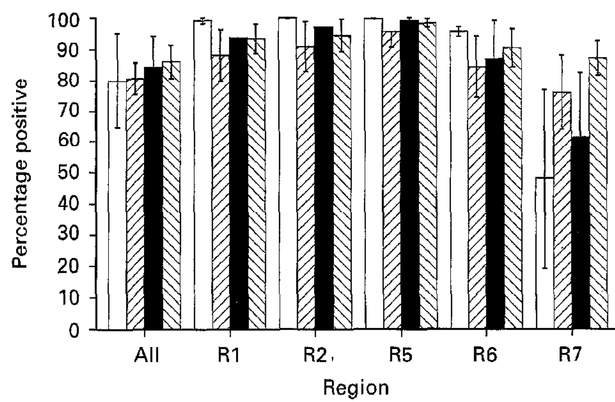

Figure 7 CD45 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

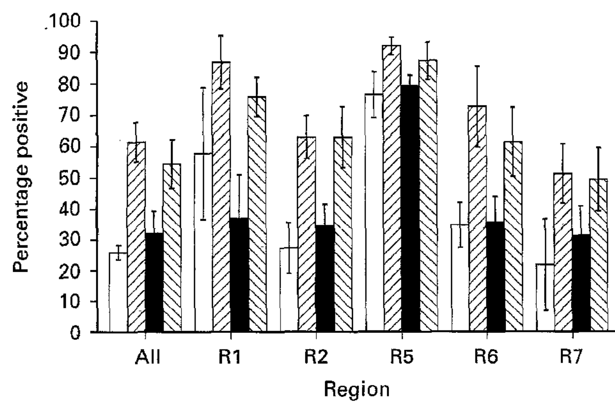

Figure 8 CD11b/c positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

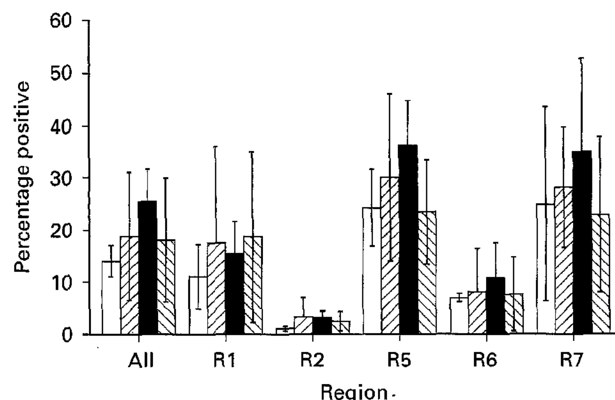

Figure 9 α/β positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

significant differences in this small population; very small numbers of R3 lymphocytes were observed with latex, and R1, R6 and R7 had small positive populations (Fig. 10). Positivity for B-lymphocyte (CD45RA) was limited mainly to R3 and R5, where no significant difference between the materials was observed (Fig. 11). The numbers of positive cells were very low, (approximately 2.0% of the total cell count.

Granulocyte G967 positivity in R1, R5, R6 and R7 was high, being generally greater than 70%, particularly for latex (Fig. 12). In R1 silicone 5 day samples had significantly lower positivity than latex 5 and 7 day and silicone 7 day samples. For R6 and R3, latex

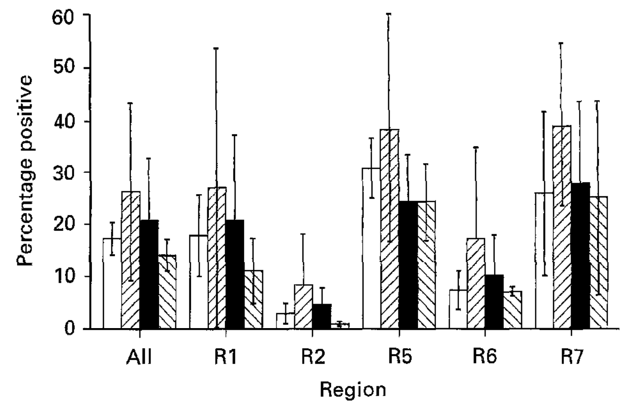

Figure 10 CD5 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

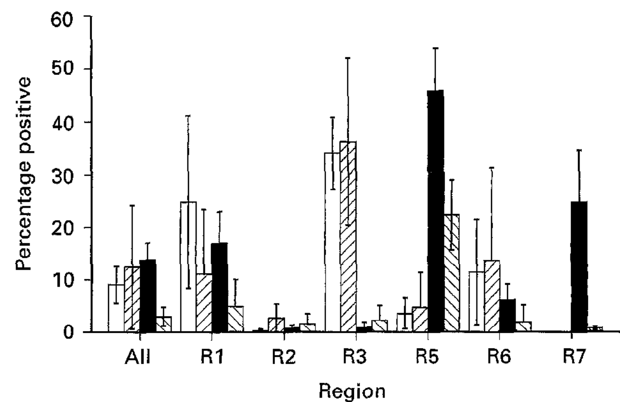

Figure 11 B-lymphocyte CD45RA positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

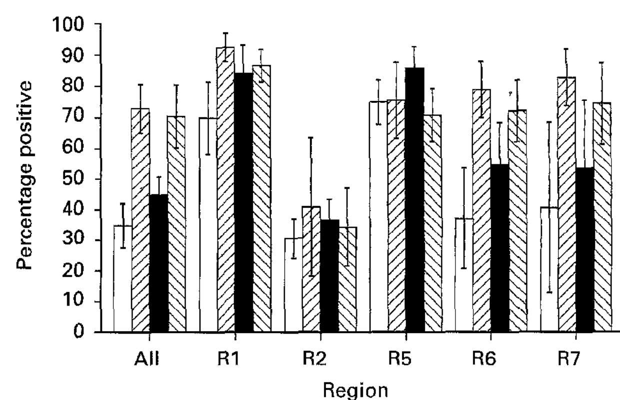

Figure 12 G967 positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

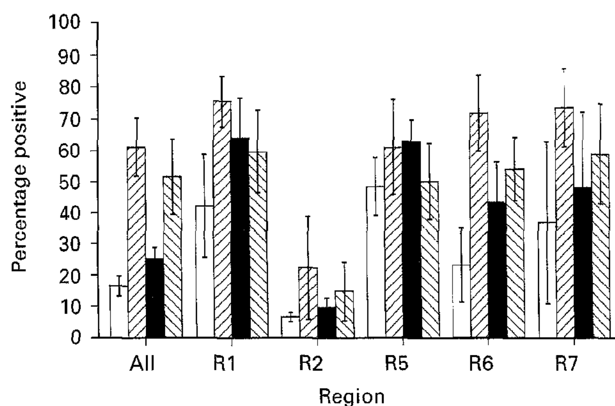

Figure 13 G149a positivity for the separated regions. □ silicone day 5; ▨ latex day 5; ■ silicone day 7; ▧ latex day 7.

5 and 7 day samples were significantly more positive than silicone 5 and 7 day samples. Also in R6, silicone 7 day positivity was significantly greater than silicone 5 day positivity. For granulocyte G149a (Fig. 13), in R6 latex 5 day positivity was significantly greater than latex 7 day and silicone 7 day positivity, which were both significantly greater than silicone 5 day positivity. No other differences were observed between these materials at the 5 and 7 day time periods.

Only one region out of the six contained cells that autofluorescenced; this was R5, which was predominantly composed of mature tissue macrophages.

4. Discussion

Significant differences were observed between the two materials at the 5 and 7 day time points with respect to granulocyte and macrophage numbers and receptor expression on these observed cells. There was a significantly larger inflammatory response for latex that involved macrophages and granulocytes of uncharacteristic size and granularity. There was some overlap in cell type within the observed regions that made cell characterization hard to resolve at this stage. The cells in R6 and R7 certainly appeared to be predominantly macrophages by FACS analysis; this was confirmed by FACS sorting then visual examination under a microscope after nuclear staining, but these regions demonstrated high percentage positivity with granulocyte markers.

The positivity for α/β and CD5 in regions other than R3, in particular R7 for latex, was complex. It was tempting to consider this as non-specific Fc receptor binding; however this was unlikely as the same cells did not have similar percentage positivities for all the monoclonal antibodies, which were of the same isotype (IgG_1) and had similar F-P ratios. It could not be ruled out that the populations of cells in R7 and R6 had some increased affinity for IgG_1 proteins. Some increased affinity IgG_1 binding responses have been reported with human immunoglobulins [7], albeit this would be slightly more involved, as there were different percentage positivities in these regions with comparable IgG_1 antibodies. Clearly this requires further investigation to fully understand the effect these materials are having on receptor expression and protein binding to interacting cells. It is interesting to note these events and that they were reproducible across this experiment ($n = 10$ for latex 7 day, and $n = 6$ for silicone 7 day).

5. Conclusion

There were significant differences in the response to silicone and latex, with large differences in antibody positivity across the panel of monoclonal antibodies. The difference in response to silicone and latex was consistent and easily proved statistically. The response in both cases predominantly involved granulocytes and macrophages with some overlap between these two cell types, with large differences between the materials, the response for latex being much more severe and involving many times more inflammatory cells. This experiment poses many questions about cell phenotype and receptor expression after exposure to an implanted material that will make future investigations very interesting. Flow cytometry is providing a powerful and reproducible technique for analysing inflammatory exudates.

Acknowledgements

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