JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 705–710

Cytocompatibility of two segmented biomedical polyurethanes

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In this work the proliferation of mitogen-activated human T lymphocytes, and human endothelial cells (HEC) adhesion and growth have been evaluated *in vitro* with two segmented polyurethanes (SPUs) having different chemical structure: Pellethane 2363-80A, a poly-ether-urethane, and Corethane 55D, a polycarbonate-urethane. The effect of the two SPUs on phytohemoagglutinin (PHA)-induced T-cells proliferation was analysed by evaluating both morphological changes of the lympho-monocyte system with scanning electron microscopy (SEM), and the perturbation of cell cycle progression with a cytofluorimetric technique. Results showed that, although the PHA stimulation of T-cells cultured on Corethane was sufficient to induce entry into the cell cycle, as evidenced by an increase in RNA content, the DNA synthesis appeared similar to that of unstimulated cultures. On the other hand, there was a significant increase of the DNA and RNA content in the culture performed in the presence of Pellethane, as in the case of PHA-stimulated control cultures. Moreover, in comparison with Pellethane, a strong decrease in monocyte attachment and spreading was observed on Corethane; with lymphocytes scattered around the monocytes, and not attached to them as in the case of Pellethane. In the experiment with HEC, the cells were directly seeded on the materials, and the number of cells attached after 4 and 72 h culture was then evaluated, the former reading being a measure for cell adhesion, the latter indicating the capacity of the cells to proliferate. Although both SPUs allowed a HEC adhesion lower than the positive control, it was possible to confirm that Pellethane well supports cell proliferation, whereas Corethane is less prone to promote cell growth

1. Introduction

The cascade of events that induces cell activation and proliferation in the presence of an implanted material remain largely undetermined, although many of the events which play key roles in the complex network of biological interrelationships leading to major cell cycle transitions have been identified. The fact that the disruption of this network is responsible for rejection of a biomaterial, and the realization that the cellular growth onto a prosthesis is probably the end point of a combination of several interactions between material surface and cell membrane, make a complete understanding of the cell cycle control, in these particular conditions, of obvious interest and importance. Furthermore, several recent observations indicate a need for an analysis of this network of growth control events using normal human cell types, despite the difficulties associated with obtaining, culturing, and analysing such biological material.

In this work it has been studied whether different materials could support cell attachment and growth. In particular, the proliferation of mitogen-activated human T lymphocytes, and human endothelial cells adhesion and growth have been evaluated *in vitro* with two copolymers from the family of segmented polyurethanes (SPUs), a class of elastomers representing an encouraging choice for the fabrication of cardiovascular devices and prostheses [1]. The selected materials were Pellethane 2363-80A, a well known poly-ether-urethane, and Corethane 55D, a recently introduced polycarbonate-urethane. Both materials are based on diphenylmethane-diisocyanate (MDI) and butanediol (BD), but differ in the composition of the soft segments (polyether, or polycarbonate), and have shown a different behaviour *in vivo*. Pellethane has been shown to undergo surface cracking after long-term implantation [2–4], whereas Corethane has been described as stable under similar conditions [5]. It was thought of interest also to study the effect of a different chemical composition of two members of the same class of polymers on cell attachment and growth under *in vitro* conditions.

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Endothelial cells have been tested because of the importance of the creation of living endothelial linings onto biomaterials surfaces which have to be directly in contact with blood [6].

Concerning the use of lympho-monocyte cultures: the T lymphocytes proliferation test has been already proposed to investigate the cell-mediated immune response in the presence of biomaterials [7]. Although this technique is well known in immunology, the purpose of the method applied to the evaluation of biomaterials compatibility has not been well described. A biomaterial can produce a perturbation on T-cells proliferation because of: (a) a direct effect on T lymphocyte activation and/or proliferation or ; (b) an indirect effect related to the interference in the cooperative action between accessory cells (or antigen presenting cells) and T-cells. It is well known that the molecules expressed on an antigen-presenting cell (APC) such as a macrophage, provide many functions in the initiation of T-cell activation [8].

In this study, we attempted to delineate the roles of the two SPUs in supporting the proliferation of human T-cells by monitoring the stimulation of these cells with the mitogen phytohemoagglutinin (PHA), which gives the most rapid and homogeneous entry into the cell cycle.

2. Materials and methods

2.1. Materials

Pellethane 2363 80AE (Upjohn) is a poly-ether urethane, obtained from poly-(tetramethylenether)glycol, PTMG and diphenylmethane-4,4'-diisocyanate (MDI), chain-extended with 1,4-butanediol; Corethane 55D (Corvita) is a poly-carbonate urethane, obtained from poly (1,6-hexyl-1,2-ethyl carbonate)diol and MDI, chain-extended with 1,4-butanediol. They were tested in the form of solvent cast sheets, obtained by dissolving Pellethane pellets in dimethylacetamide (DMAc), or appropriately diluting the original Corethane solution in DMAc, then completely evaporating the solvent under vacuum at 60 °C. The sheets were sterilized with γ rays at a dose of 0.4 Mrad (Gammatom Spa, Guanzate, Como, I). Corethane was kindly provided by Corvita Co., Miami, FL.

2.2. Human lymphocytes proliferation test

2.2.1. Lympho-monocyte cultures

Human lymphocytes and monocytes were separated from vein blood on ficoll-paque gradient (Lymphoprep). The blood directly stratified on Lymphoprep was centrifuged for 30 min at 200 × g; the cells lying at the interface were taken off, washed twice with PBS, centrifuged, and resuspended in medium RPMI 1640, supplemented with 10% foetal calf serum and antibiotics. The lympho-monocyte suspension was then stratified onto the polyurethane samples, or placed in the wells of polystyrene culture plates (Falcon). The experiments were made in triplicate. After 2 h, the mitogen phytohemoagglutinin(PHA) was added, in order to stimulate T-cells to divide. After 48 h incubation in a 5% CO_2 humidified incubator, some material samples were taken off, and fixed with glutaraldehyde for scanning electron microscopy (SEM) analysis (see 2.2.2). Other samples were extensively washed with pre-warmed PBS, and the lymphocytes collected for cell cycle analysis (see 2.2.3).

2.2.2. Optical and scanning electron microscopy (OM and SEM) observations

OM analysis was made with an Olympus IMT-2 microscope on unfixed samples at different times of culture.

For SEM analysis, the samples were fixed with a 2.5% glutaraldehyde solution in 0.1 M cacodilate buffer (pH 7.2) dehydrated through a graded series of ethanol (up to 100%), and critical point-dried using CO_2. Specimens, mounted on aluminium stubs, were sputter-coated with gold, and examined with a Leica-Cambridge Stereoscan 440 microscope at a 3–7 kV acceleration voltage.

2.2.3. Cell cycle analysis

Cell cycle progression was analysed with a flow cytometer methodology. Briefly, to 100 µl culture medium containing $1-2 \times 10^6$ cells 200 µl of a solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.8 M HCl were added. After a 2 min incubation on ice, 600 µl of a buffer containing 0.56 M citric acid, 0.88M Na_2HPO_4, 1 mM EDTA, and 50 µM acridine orange (pH 4.4) were added, making the final pH = 3.8. After an additional 20 min incubation on ice, the cells were fixed by the addition of 80 µl of 37% formaldehyde. The cells were analysed with the FACS II (Becton, Dickinson Cytofluorimeter) at 488 nm excitation. Under the conditions used, green fluorescence (520–540 nm) provides a measure of DNA content, and red fluorescence (> 620 nm) of RNA content.

2.3. Human endothelial cells (HEC) adhesion and growth test

HEC were isolated from umbilical vein by treatment with collagenase, and cultured at 37 °C in medium 199 with 20% new-born calf serum, heparin, penicillin, streptomycin, and fungizone as already described [9]. The medium was changed twice a week; HEC were passaged (split ratio 1 : 3) once a week, and used within the fifth passage. Polyurethane discs cut from the solvent cast sheets and having the same diameter as the wells of 24-well tissue culture plates were placed on each well. HEC suspensions were directly seeded onto the wells, some of them being free of any disc to act as positive control, The negative control was represented by an aged sample of another segmented polyurethane (here indicated as TPX), able to inhibit cell proliferation. After 4, or 72 h, the cells were washed twice with Ca^{++}- and Mg^{++}-free phosphate buffer saline (PBS), detached by short exposure to trypsin/EDTA, then counted in a hemocytometric chamber.

3. Results

3.1. Human lympho-monocytes cultures

In this experiment we elected to analyse the effects of the tested polyurethanes on phytohemoagglutinin-induced T-cells proliferation by evaluating both the morphological changes of the lympho-moncyte cell system, and the perturbation of cell cycle progression in terms of DNA, and RNA content of T lymphocytes.

3.1.1. Optical and scanning electron microscopy analysis

Using optical microscopy, in the case of Pellethane it was possible to detect a number of lymphocyte clusters tightly contacting monocyte-macrophage cells, and indicating an active proliferation of the lymphocytes; in contrast, the cultures on Corethane showed a reduced number of cell aggregates, and thus a lower ability of lymphocytes to proliferate. Fig. 1a and c shows the images of Pellethane and Corethane, respectively, at 48 h culture.

SEM analysis was performed to document changes of cell surface morphology resulting from both monocyte attachment and activation, and lymphocyte proliferation obtained by culturing blood mononuclear cells on Pellethane, and Corethane (culture polystyrene as control). At low magnification (Fig. 1b, and d) the cell distribution on the materials surface after PHA stimulation seemed to be affected by the type of polymer used. Dense clusters of lymphocytes are seen distributed unevenly over the surface of Pellethane, and the control, with fairly high cell concentrations. In the presence of Corethane the number of clusters was significantly decreased with a large surface area with no cells at all. Moreover, on Corethane the lymphocytes were usually observed scattered around the monocytes, and not attached to them as in the case of Pellethane and control polystyrene cultures. Finally, Corethane showed many rounded monocytes with low cytoplasmatic spreading, whereas Pellethane and the control showed various elongated and flattened monocytes, with numerous filopodia as attached anchors (Fig. 2a–d).

3.1.2. Pertubation of cell cycle progression

The analysis by the fluorescence-activated cell sorter indicated that human T-cells incubated in the medium were a homogeneous population with respect to two parameters associated with the resting, or GO state: a 2N content of DNA, and a relatively low amount of RNA per cell (Fig. 3a). The mean fluorescence (MF) at > 620 nm, a value proportional to the RNA content, was 35 units for the unstimulated control sample. Upon stimulation with PHA, at a concentration shown to be optimal for triggering T-cells proliferation, there was a significant increase of the DNA and RNA content (MF = 70 units) in the control culture, and in the culture performed in the presence of Pellethane (Fig. 3d and c). On the other hand, the presence of Corethane permitted a significant increase in the cellular RNA content (MF = 67 units) to levels

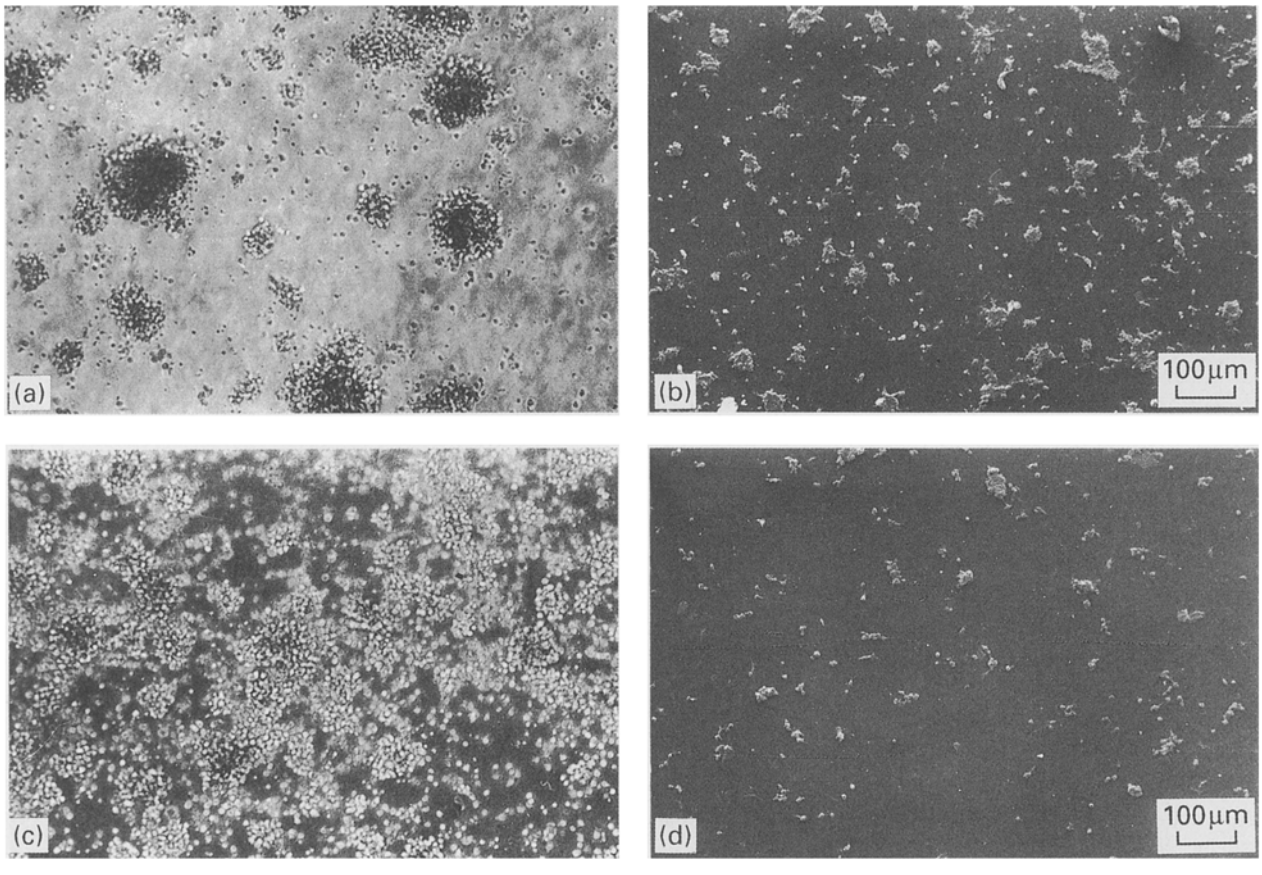

Figure 1 Optical microscopy (1a, and c) and SEM analysis (1b, and d) of 48 h lymphomonocyte cultures allowed to proliferate on Pellethane (a, and b) and Corethane (c, and d) surfaces. Low magnification.

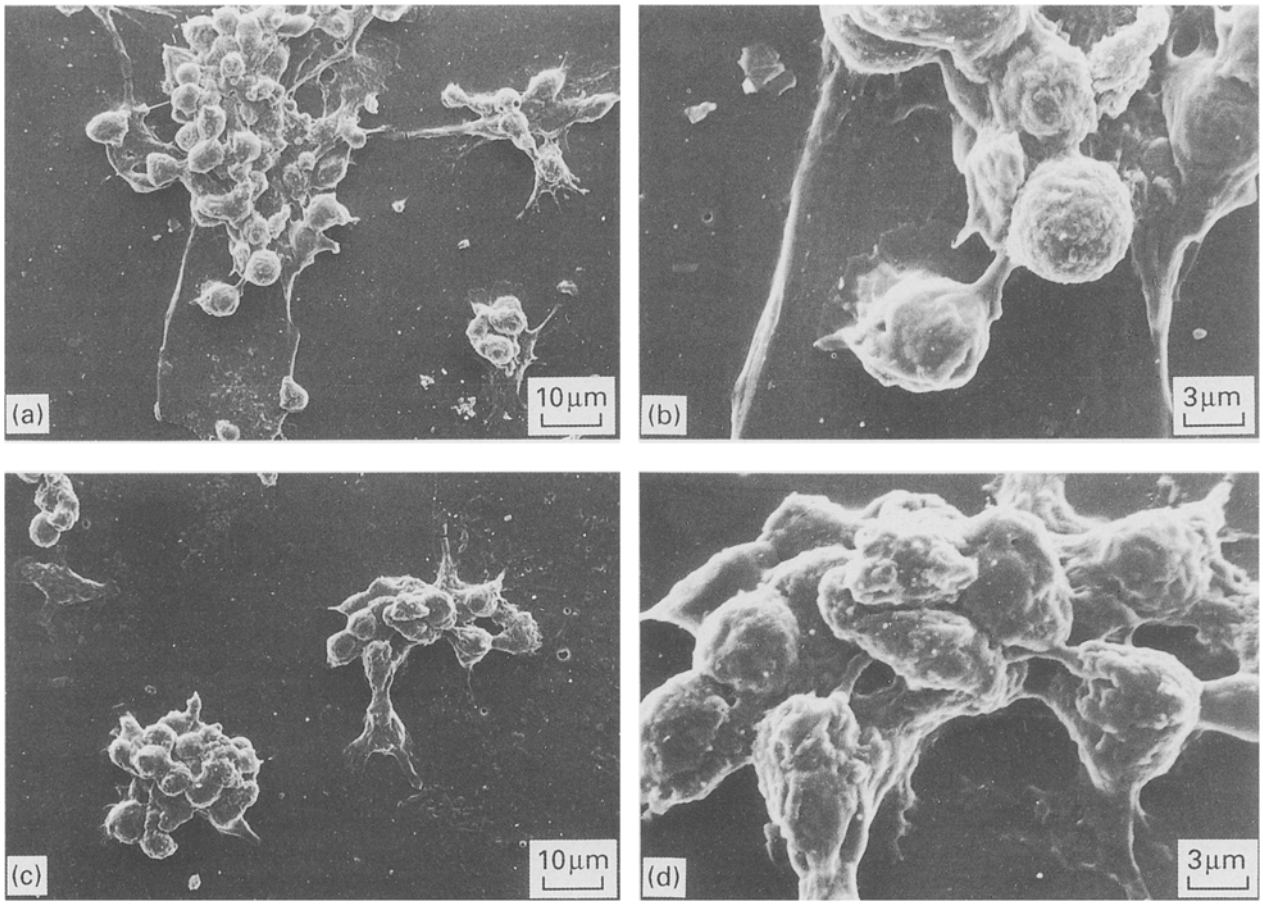

Figure 2 SEM analysis of 48 h lympho-monocyte cultures allowed to proliferate on Pellethane (a, and b) and Corethane (c, and d) surfaces. High magnification.

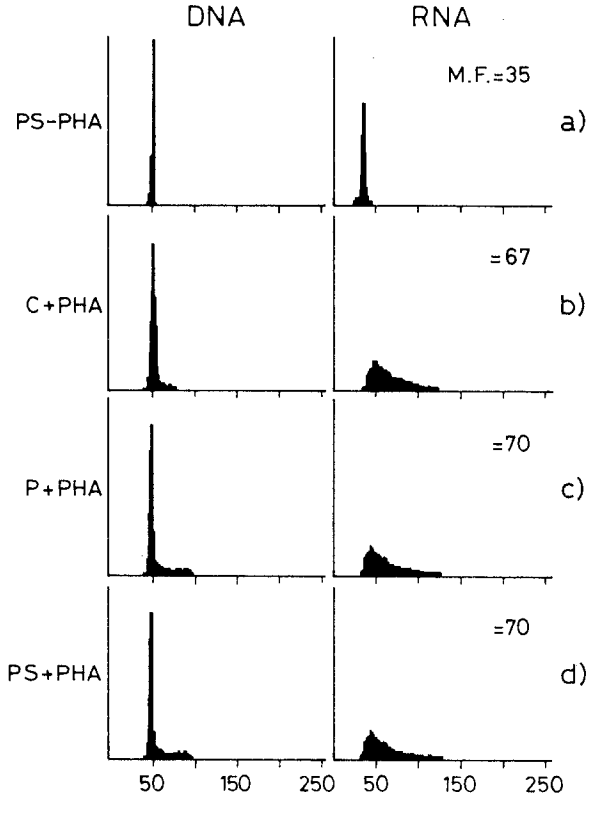

Figure 3 Cell cycle analysis of T-cells cultured in RPMI complete medium on control polystyrene (PS), or Corethane (C), or Pellethane (P) surfaces. Human peripheral mononuclear cells were incubated for 48 h in absence (− PHA), or in presence (+ PHA) of the mitogen phytohemoagglutinin (PHA).

approaching that of proliferating cells, but still no DNA synthesis occurred (Fig. 3b). These findings were confirmed by measuring the incorporation of [^{3}H] thymidine into DNA and [^{3}H] uridine into RNA (data not shown).

3.2. HEC adhesion and growth

In the experiment with human endothelial cells, the cells were directly seeded on the materials, and the number of cells attached at 4 and 72 h was then evaluated. The former reading (at 4 h) gives data on the ability of the test materials to promote cell adhesion, the latter indicate the capacity of the cells to proliferate on the materials. As shown by data reported in Fig. 4, both Corethane and Pellethane show a similar ability to promote HEC adhesion on their surface 4 h after seeding, but are poor substrata for cell attachment when compared with the corresponding control (Control B in Fig. 4). However, they are not cytotoxic, since the number of cells does not decrease 72 h after seeding. Furthermore, Pellethane stimulated the proliferation of adherent cells, as demonstrated by the increased number of them at 72 h. This finding is in agreement with our previous results [9]. In contrast, for the negative control (a degraded, and thus toxic, polyurethane), the number of adherent cells is very low after 4 h, and dramatically decreases to zero 68 h later, suggesting cell toxicity and death.

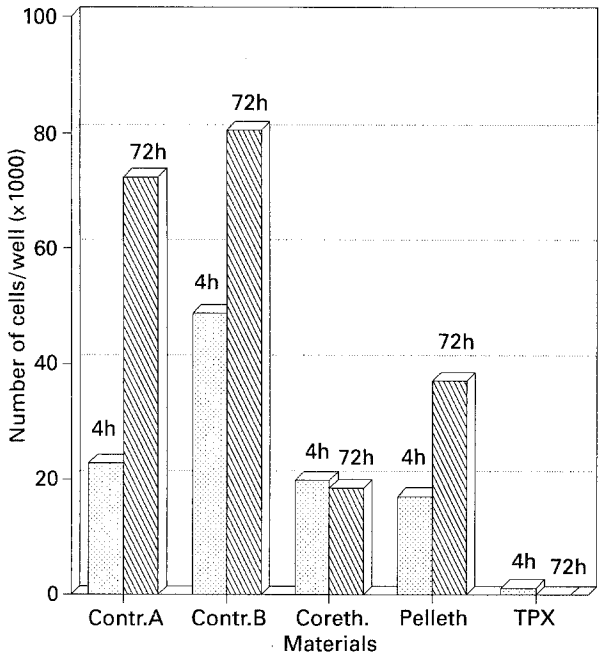

Figure 4 Human endothelial cells adhesion (dotted column: 4 h after seeding) and growth (oblique line column: 72 h after seeding) onto the tested materials. Control A (Tissue culture plastic: 30 000 cells/well seeded); Control B (Tissue culture plastic: 60 000 cells/well seeded). On the other materials: Coreth. (Corethane 55D); Pelleth. (Pellethane 2363 80AE); and TPX (an aged, and degraded segmented polyurethane) 60 000 cells/well were seeded. (Average values of duplicates, sd values within 10%.)

4. Discussion

In this work the ability of two chemically different segmented polyurethanes (Pellethane 2363-80A, and Corethane 55D) to support (a) T-cells proliferation and (b) human endothelial cells adhesion and growth, has been considered:

In particular, some aspects of the proliferation of human T lymphocytes in the presence of the selected SPUs were investigated in an *in vitro* culture system, attempting to delineate the role of these materials in supporting the proliferation of these cells by monitoring their stimulation with the polyclonal mitogen phytohemoagglutinin (PHA), which gives the most rapid and homogeneous entry into the cell cycle.

As previously noted, T-cells can be activated by PHA to proliferate only in the presence of accessory cells, such as monocyte/macrophages. The costimulatory function of these cells has been revealed primarily in studies involving the response of resting G_0 T-cells that have been depleted of antigen-presenting cells (APCs) contained in the adherent cell population. These purified resting T lymphocytes fail to produce interleukin-2 (IL-2), and to proliferate in response to mitogenic lectins such as PHA, although the degree of cell activation was demonstrated by an increase of RNA content in the cells, and the appearance of IL-2 receptors and transferrin receptors on their membrane [10].

Also in our experiments, although the PHA stimulation of T-cells cultured on Corethane was sufficient to induce entry into the cell cycle, as evidenced by an increase in RNA content (near doubling in mass of the total cellular RNA of quiescent cells), a necessary prerequisite for the G1 to S phase transition, the DNA synthesis appeared similar to that of unstimulated cultures. Even though the possible role of Corethane in mediating these effects can only be speculated upon, this material induced three major changes in T-cell cultures: (1) RNA levels increased to those characteristic of cells in very late G1 phase; (2) no appearance of DNA replication; and (3) a strong decrease in monocyte attachment and spreading on to the material surface.

It is likely that the last event (absence of monocyte spreading and peripolesis) could be considered as the mirror of an inefficient monocyte activation with their relatively inability to behave as accessory cells for T-cell proliferation. Indeed, SEM analysis suggest that, although the materials examined in this study provide an effective contacting surface to which monocytes can adhere and become activated, there is a difference in the efficiency with cells attached to the different polyurethanes. Cell attachment usually may lead to an activation state, as demonstrated by morphological changes of monocytes adherent on Pellethane, and polystyrene (as control material). By SEM analysis, these morphological changes, were cellular spreading associated with flattening, the presence of various filopodial extensions, increased cell size, and increased plasmamembrane ruffling. On the other hand, cells attached to Corethane remained very rounded, showing little cytoplasmatic spreading. According to the intensity of cell activation observed by SEM on control polystyrene and Pellethane surfaces, it is possible to correlate the greater densities of morphologically activated monocytes with the higher levels of DNA synthesis. The decreased monocyte adhesion and activation on the Corethane surface was well reflected in the low DNA replication of T lymphocytes.

From the experiments with human endothelial cells, it was possible to confirm that Pellethane, as observed in a preceding paper [9], well supports cell proliferation whereas Corethane is less prone to promote cell growth.

5. Conclusions

Our experiments indicate that monocyte adhesion to polymer surfaces is of major importance in terms of biocompatibility. Adherence, as well as the subsequent cell activation, can lead to stimulation of cell accessory functions. This event may have profound effects on the lymphocyte host response to biomaterials. On the other hand, the ability to encourage endothelial cells adhesion, and promote their growth represents an ideal challenge for materials to be used in direct contact with blood.

Materials that are well tolerated *in vivo* and promote cell adhesion on their surfaces are biocompatible and bioactive (a benign reactivity implying a reactivity appropriate to the intended use); on the other hand, a failure of cells to spread or attach to a surface does not necessarily indicate poor biocompatibility. Materials which do not interfere with cell adhesion and growth (or activation) may be considered as biocompatible

but inert. From these points of view, Pellethane behaves as a bioreactive material, whereas Corethane can be considered much more bioinert.

Acknowledgements

This work is supported by Italian C. N. R. (Progetto Finalizzato Chimica Fine II, Progetto Strategico "Icaros", Progetto Speciale Biomateriali). The tests with endothelial cells were carried out at the Vascular Biology Laboratory of Mario Negri Institute for Pharmacological Researches (Milano, Italy).

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