Arch Immunol Ther Exp, 2004, 52, 129–136
PL ISSN 0004-069X

WWW.AITE–ONLINE.ORG
Original Article

Received: 2003.08.05
Accepted: 2004.01.16
Published: 2004.04.15

Effect on peripheral blood natural killer cytotoxic cell activity in rats after intraperitoneal implantation of double veloured polyester (Dacron) prosthesis

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Source of support: self financing.

Summary

Introduction: The aim of this study was to assess the changes affecting natural killer cytotoxic cell (NKCC) activity following intraperitoneal implantation of a double veloured polyester prosthesis in a rat model.

Materials and Methods: Blood samples were taken by cardiac puncture 1 h before (base line) and 14, 28, 100 and 180 days post-implantation. Peripheral blood mononuclear cells were separated from heparinized blood by density centrifugation. A standard, 4 h ^{51}Cr-release assay against YAC-1 target cells at effector to target ratios of 12:1; 25:1 and 50:1 was performed and the number of total leukocytes, lymphocytes, granulocytes, monocytes, and large granular lymphocytes (LGLs), as well as serum corticosterone levels (radioimmunoassay method) were determined.

Results: Comparative analysis of the results obtained from animals with implants, baseline samples, and a control group (laparotomy only) revealed lower NKCC, LGL, leukocyte and lymphocyte counts and elevated plasma corticosterone levels in animals receiving the implant on the 14th day post-implantation.

Conclusions: Our findings indicate that the polyester implant can transiently modulate immune system activities. Since NK cells are important in the control of viral infection and carcinogenesis in humans, it is possible that the stress generated by polyester prostheses can exacerbate the surgical stress and put patients at a higher risk for viral infection and/or metastases.

Key words: **Dacron • NKCC • LGL • rat • lymphocytes • leukocytes**

Full-text PDF: http://www.aite-online/pdf/vol_52/no_2/5242.pdf

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INTRODUCTION

The use of artificial materials in surgery is becoming more common. However, recent research data have indicated that such implants are not completely inert, as. they affect the proliferation of natural killer cytotoxic cells (NKCC)[3, 8, 12, 15, 49] and thus modulate the immune response.

Natural killer (NK) cells are the only group of cytotoxic cells that can proliferate and kill virus-, bacterium-, or protozoon-infected cells without need for antigen presentation by the major histocompatibility complex or coating of the target cells by antibodies[38, 42]. They are also immunomodulatory cells since they excrete many cytokines and take part in the early stages of erythropoiesis[18]. These lymphocytes are sensitive to stress factors generated by surgery[6, 35, 40] and anesthetics[4, 5]. Decreased activity of NKCCs usually leads to an increase in viral infection and the risk for tumor growth and metastases[4, 5, 6, 24, 30].

Dacron is commonly used in cardiac, vascular, and other surgery procedures as a relatively inexpensive textile prosthesis[48, 54]. Many synthetic prostheses, including Dacron, induce an inflammatory reaction[19, 50, 52] and the release of stimulatory or inhibitory cytokines that act on natural killer cytotoxic cells[7, 9, 41]. A survey of the literature did not reveal any data on the *in vivo* effect of Dacron implants on NKCCs. Therefore, we conducted this study to assess what effect the implantation of Dacron implants in a rat model might have on the immune system by focussing on changes observed in NKCCs, large granular lymphocyte (LGLs), which are a morphological subset of NK cells[2, 10, 33], the total numbers of leukocytes, lymphocytes, granulocytes and monocytes, as well as the level of plasma corticosterone.

MATERIALS AND METHODS

Animals

The study was conducted on 20 male Wistar rats, weighing approximately 220–260 g at the beginning of the trial. The animals were housed in individual cages with free access to standard food and water, under a 12 h light/12 h dark illumination cycle.

Blood sampling

Blood samples (2 ml) were taken from all the animals by cardiac puncture (under diethyl ether anesthesia). Each rat was placed in a glass chamber saturated with ether vapor. When the animal was anesthetized, it was removed from the chamber and a needle connected to a syringe was inserted into the ventricle by puncture of the chest. The whole procedure took 1–2 min. The samples were collected one hour before surgery (basal) and on the 14th, 28th, 100th and 180th day post-implantation. The collection time was between 8.00 and 9.00 a.m., which corresponded to 2–3 h after light onset.

Surgery

The surgery was performed under thiopental sodium anesthesia (20 mg/kg intraperitoneal). After basal sampling, the rats were randomly divided into two groups of 10 animals each and operated. A rat was mounted on its back on the operating table. The abdominal skin was shaved, sterilized with iodine, and a 0.5 cm-long incision was made 3 cm below the sternum in the middle line of the abdomen. The experimental animals were implanted in the abdominal cavity with a 1-cm^2 patch of sterile double veloured polyester prosthesis (Dacron), which is commercially available as Kardioplast H. Laparotomy (incision only) was performed on the control group. Two catgut stitches closed the wound, which was then covered with crystalline penicillin.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by density centrifugation (Gradisol L, Poland). After centrifugation, the isolated cells were collected with a Pasteur pipette, washed 3 times with phosphate-buffered saline, counted, and suspended in complete medium RPMI 1640 (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml; GibcoBRL, USA). Subsequently, the PBMCs were incubated on plastic Petri dishes for 120 min at 37°C. Adhered cells were removed and non-adherent mononuclear cells were collected and adjusted to $1x10^7$ cells/ml in complete medium. These cells were used as effector cells for NKCC determination.

NKCC assay

The cytotoxicity of NK cells was quantified using a ^{51}Cr-release assay. The labeling of target cells (YAC-1) was as previously described[51]. The labeled target cells (T) were cultured for 4 h in sterile round-bottomed micro-well plates (ProLab, Poland) using various amounts of effector cells (E) vs. a fixed amount of target cells. The total volume of the mixture was 200 μl and the E:T ratios were 50:1, 25:1, and 12:1. Each experiment (Exp) was performed in triplicate

under standard culture conditions. Spontaneous ^{51}Cr--release wells (Sp) had target cells plus 100 µl of complete medium. Maximum release wells (Max) contained target cells plus 100 µl of complete medium with 5% Triton X-100 (Serva, Germany). The assay was terminated after 4 h by centrifuging the plates. Subsequently, 100 µl of supernatant was removed from each well and the samples were counted in a MiniGamma counter (LKB, Finland) for 1 min. Cytotoxicity percentages were calculated as [(Exp--Sp)/(Max-Sp)] × 100. Since the differences between the experimental data were statistically significant except for the E:T ratio of 25:1, this ratio was used (Fig. 1) for period results presentation.

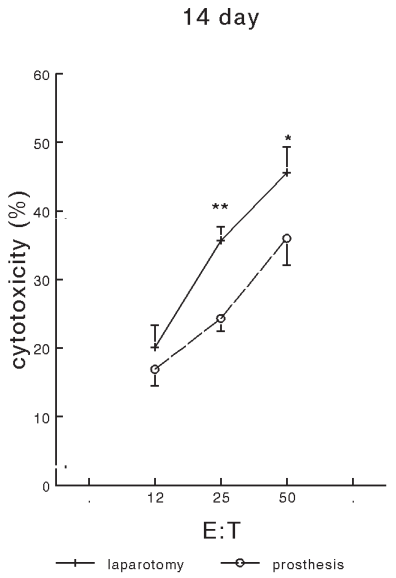

Figure 1. Comparison of the NKCC counts of the laparotomy and prosthesis implant groups on the 14th day postoperative at all examined E:T ratios. * $p<0.05$, ** $p<0.01$.

Morphologic analysis of LGLs

The morphologic analysis of LGLs was performed as previously described[51] with some modifications. The LGL cells were prepared by centrifuging 0.2×10^6 PBMCs for 10 min in 0.2 ml of complete medium and spread on microscope slides, using a Cytocentrifuge Sigma 3–15 (Germany). After air-drying, the cells were stained with Giemsa and May Grünwald solutions (Pappenheim method). The slides were analyzed by microscopy under oil immersion.. At least 200 cells were examined on each slide and the percentage of LGLs was determined. The absolute number of LGLs was calculated by multiplying the total number of lymphocytes counted by the percentage of LGLs/100.

Leukocyte, lymphocyte, granulocyte, and monocyte counts

The absolute number of leukocytes was determined using a Celldyna 400 (Abbott, USA). The percentages of lymphocytes, granulocytes, and monocytes were determined microscopically using whole blood smears stained with Giemsa and May Grünwald solutions (Pappenheim method). The absolute numbers of lymphocytes, granulocytes, and monocytes were calculated by multiplying the total number of leukocytes counted by the respective percentages of lymphocytes, granulocytes, and monocytes/100. All absolute cell numbers were expressed as the number of cells per 1 µl of blood.

Determination of the concentration of plasma corticosterone

Plasma corticosterone concentration was measured by radioimmunoassay using a commercially available kit (Rat corticosterone [^{125}I], Biotrak Amersham, England) and a Mini Gamma counter (LKB, Finland).

Data analysis

The results were analyzed using the Kruskal-Wallis one-way analysis of variance and the Wilcoxon test for unpaired cases. The significance threshold was $p \le 0.05$. The obtained data are presented as mean ± standard error.

RESULTS

NKCCs

NKCC counts in the control group (laparotomy only) were found to be significantly affected by post-injury time ($p<0.05$). On the 14th day after surgery, NKCC counts in the control group were significantly different from those in the group receiving a prosthesis ($p<0.01$ at E:T=25:1 and $p<0.05$ at E:T=50:1; Fig. 1). A comparison with basal NKCC activity (27.65% ± 1.80) showed that animals in the control group (E:T=25:1) had significantly enhanced NKCC counts on the 14th day after surgery (35.65% ± 2.06, $p<0.05$; Fig. 2), while rats with polyester prosthesis showed a slight depression in NKCCs (24.26% ± 1.84). In the remaining post-surgery periods there were no significant differences between the two groups, nor was any difference found with basal activity.

LGL number

Analysis of variance showed that both the laparotomy ($p<0.01$) and prosthesis ($p<0.05$) groups were

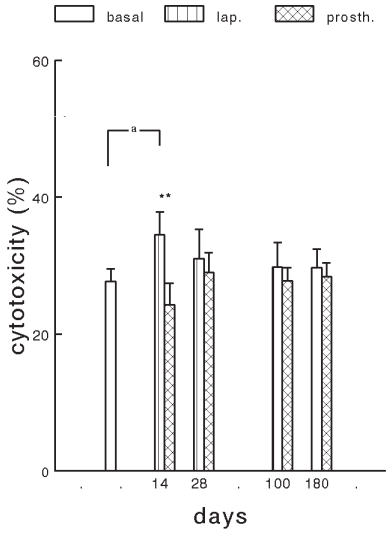

Figure 2. Influence of prosthesis implantation on peripheral blood NKCC activity at E:T=25:1. Basal – activity 1 h before surgery, lap. – rats after laparotomy, prosth. – rats after prosthesis implantation. [a]p<0.05 (comparison between the baseline and post-surgery data), **p<0.01 (comparison between the laparotomy and prosthesis implant groups).

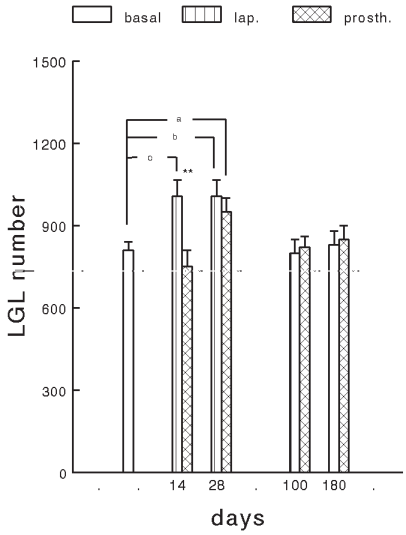

Figure 3. Influence of prosthesis implantation on peripheral blood LGL number. [a]p<0.05, [b]p<0.01, [c]p<0.001 (comparison between baseline and post-surgery data), **p<0.01 (comparison between the laparotomy and prosthesis implant group).

significantly affected. Rats receiving a prosthesis showed a slightly diminished LGL number on the14th day post-surgery compared with pre-surgery (750 ± 60 vs. 810 ± 30; Fig. 3). However, the difference was significant compared with the laparotomy group (1070 ± 60, p<0.01). On the 28th day post--implantation, both groups differed significantly from the baseline (950 ± 50 vs. 810 ± 30, p<0.01). The number of LGLs in the laparotomy group differed from that of the baseline on the 14th (1070 ± 60 vs. 810 ± 30, p<0.001) and 28th day (1060 ± 60 vs. 810 ± 30, p<0.01) post-operation.

Cell count and corticosterone level results

Leukocytes. Analysis of variance indicated that during the experiment the number of leukocytes was only affected in the control group (p<0.001). On the 14th day after implantation, the number of circulating leukocytes in animals with prosthesis did not change significantly from base-level values (13500 ± 800 vs. 14000 ± 500). However, there was a significant difference in the control group (13500 ± 800 vs. 19100 ± 900, p<0.001; Table 1).

Table 1. The peripheral blood white cells numbers and serum corticosterone level 1 h before (basal) and at different days after surgery in the laparotomy (lap.) and prosthesis implant (prosth.) groups

	Basal	Groups	Days			
			14	28	100	180
Leukocyte number (×10^3)	14.00±0.50	Lap.	19.10±0.90[c]***	15.50±1.10	13.50±0.70	14.00±1.00
		Prosth.	13.50±0.80	15.00±0.80	14.30±0.90	14.10±0.90
Lymphocyte number (×10^3)	10.80±0.60	Lap.	14.10±1.10[b]**	12.60±1.30	11.10±1.10	10.80±0.70
		Prosth.	9.00±1.00	11.30±1.10	11.10±1.00	11.00±1.10
Granulocyte number (×10^3)	2.70±0.20	Lap.	4.50±0.50[c]	2.50±0.30	2.70±0.10	2.60±0.20
		Prosth.	4.10±0.40[a]	2.50±0.30	2.70±0.50	2.60±0.70
Monocyte number (×10^3)	0.55±0.05	Lap.	0.51±0.04	0.40±0.04	0.52±0.03	0.60±0.06
		Prosth.	0.40±0.05	0.70±0.06	0.53±0.06	0.52±0.05
Corticosterone (ng/ml)	31.30±2.90	Lap.	27.70±2.70**	26.20±3.10	33.80±2.40	32.60±3.00
		Prosth.	46.30±3.40[b]	22.80±3.20	28.90±4.40	30.10±2.40

[a]p<0.05, [b]p<0.01, [c]p<0.001 (comparison between the baseline and post-surgery data), **p<0.01, ***p<0.001 (comparison between the laparotomy and prosthesis implant groups).

Lymphocytes. The analysis of variance indicated that during the experiment lymphocyte number was significantly affected only in the control group ($p<0.05$). On the 14th day post-operation, animals with prosthesis had a slight decrease in lymphocyte number compared with basal data (9000 ± 1000 vs. 10800 ± 600). The control group had lymphocytosis (14100 ± 1100, $p< 0.01$). Therefore, both the groups differed significantly ($p<0.01$).

Granulocytes. Analysis of variance indicated that during the experiment granulocyte number was significantly affected both in the control ($p<0.01$) and the experimental group ($p<0.05$). Both groups reacted with significant granulocytosis compared with basal data (2700 ± 200 and 4100 ± 400 vs. 4500 ± 500, $p<0.05$ and $p<0.001$, respectively). There were no significant differences between the control and the experimental groups.

Monocytes. The number of monocytes was not significantly affected by post-injury time in both groups. There were also no significant differences between the control and the experimental groups.

Corticosterone level. Analysis of variance indicated that during the experiment the corticosterone level was only affected in animals with the prosthesis ($p<0.001$). Prosthesis implantation enhanced the corticosterone level on the 14th day post-operation compared with the baseline (46.30 ± 3.40 ng/ml vs. 31.30 ± 2.90 ng/ml, $p<0.01$). The control group did not show significant changes. On the 100th and 180th day post-surgery, no significant differences were observed in any of the cell numbers nor in the corticosterone level (Table 1).

DISCUSSION

It is very difficult to investigate the very early influence of prosthesis implantation on immunity because a strong depression of the immune system is always observed during and after surgery. The extent of this depression depends on the extent of the surgery itself and the kind of anesthetics used [1, 34, 54]. Anesthetics administered via vein or peritoneum lead to a depression in the immune system, including NK cell activity. However, after 24 to 48 h[4, 5] and up to the 7 days of depression, the activity returns to baseline activity[44]. To minimize the effect of surgerical procedures on the immune system we used the anesthetic thiopentone sodium to minimize NKCC depression[24] and made a small incision (5 mm long). It is known that the extent of surgery correlates with immunological depression[1]. Since antibiotics administered during surgery can also exert some effects on the immune system, and may drastically change the results of the experiment[26], we applied antibiotics over the wound. Further, we checked that diethyl ether anesthesia did not cause any major changes in the NKCC and leukocyte numbers and in the serum corticosterone level[23].

To exclude effects non-specific to the experiment that may be generated by surgery stress alone, we started investigating the effects of prosthesis implantation on NKCCs 14 days after surgery.

The animals reacted differently after laparotomy and prosthesis implantation: laparotomy showed NKCC stimulation correlating with general leuko- and lymphocytosis, while prosthesis implantation showed diminished NKCCs at that time.

Although the surgery was done aseptically, the increase in NK cytotoxic activity, observed in the control group on the 14th and 28th days, could have been caused by infection and/or inflammation. Investigations have indicated that one of the factors that could activate NK lymphocytes could be their interaction with bacteria or products of their metabolism[20, 43].

Other results showed that the increase in NK cell activity observed after surgery (up to 7 days) correlated with the increase in LGL cells, which are a morphological subset of NK cells[22, 42]. Our results indicate that the changes observed in NKCC are also accompanied by alternations in the number of peripheral blood LGLs.

The increase in NKCCs observed in the control group may be a result of the mobilization of NK cells from the extravascular space, spleen or lymph nodes into the circulation[46].

The leukocytosis observed on the 14th day after the surgery in the control group was caused by significant lymphocytosis and granulocytosis in response to infection or inflammation. Shortly after surgery, a significant increase in the number of neutrophiles[53], as well as their activity[39], was observed. This process is an obvious consequence of the surgery. Granulocytes and mononuclear cells are recruited to the site, and these cells, as well as local fibroblasts and endothelial cells, are stimulated to release cytokines into the peripheral blood circulation[47]. Some of these cytokines[43] could modulate NK cell activity[20, 43]. The main modulators of the immune system cells produced in surgical stress are interleukin (IL)-1, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α and β, and interferon α[13, 16, 28, 31, 53].

Current results show that Dacron is not immunologically inert. The immune system of rats with a prosthesis undergoes a slight "anergy" shortly after implantation. We expected that the immune system of animals with the prosthesis would be similarly or more stimulated than that of the animals in the control group. However, we found that on the 14th day after implantation of the prosthesis, the experimental animals showed a slight decrease in NK cell activity compared with the baseline and a significant difference compared with the control group. The results also showed a slight decrease in the number of LGLs, leukocytes and lymphocytes. A significant increase in plasma corticosterone levels compared with the control group was also observed.

A synthetic prosthesis activates macrophages and induces acute inflammatory reaction[19, 50, 52] as well as the release of cytokines[7, 9, 41], which could strongly influence NKCCs[10, 13, 46].

In vitro incubation of white blood cells with Dacron induces them to secrete significant amounts of IL-6 and TNF-α[41]. IL-6 exerts an activating influence on the hypothalamo-pituitary-adrenocortical (HPA) axis. In the hypothalamus, these cytokines increase the release of corticotropin-releasing hormone (CRH)[32], which decreases NK cell activity[21]. The evidence also suggests that IL-6 enhances the synthesis of glucocorticoids and their release from the adrenal gland[45]. The corticosterone level rises in experimental animals on the 14th day after implantation. It has been known for a long time that the normal feedback inhibition of the HPA axis by glucocorticoids does not occur during stress[47]. Thus, despite high levels of circulating glucocorticoids, immune system inhibitory hormones such as CRH and adrenocorticotropic hormone continue to be secreted, but feedback inhibition does not occur despite the prolonged stress[25]. Therefore, the effects observed 2 weeks after implantation can be treated as one of the factors prolonging surgery stress. The transient depression in NKCCs, the slight decrease in lymphocyte number, and the granulocytosis observed on the 14th day after implantation can be caused either by a higher level of CRH or/and by glucocorticosteroids[11]. The slight changes observed could be a result of a slight but significant elevation of corticosterone. Only strong stress, where corticosterone rises more than 100%, could result in a significant decrease in NKCCs, lymphopenia and, granulocytosis[11].

The observed reaction could also be specific to prosthesis implantation. Macrophage activity can be one of the factors causing the significant decrease in NKCCs seen on the 14th day after implantation and its slight increase on the 28th day. A polyester prosthesis induced intense acute inflammatory reaction on the 3rd day, which gave way to a typical chronic response after the 4 week[29]. In a pig model, Dacron significantly enhanced IgG level on the 10th, 17th, 24th and 62nd days after implantation[54]. In the case of double veloured Dacron implants, the peak of implant infiltration by host cells (mostly macrophages) was observed in the period between 2 and 3 weeks[36]. Compared with other types of implants, Dacron is more inflammatory. The perigraft tissue is infiltrated with different types of macrophages[17]. Acute non-septic inflammation also inhibits NKCCs and enhances metastatic development[14]. Since a partial restoration of NKCCs was obtained after depletion of plastic-adherent cells (mostly macrophages), suppressor macrophages may be involved in NKCC suppression[17]. Removing all macrophages from the buffy-coat mononuclear cells significantly reversed the NK cell suppression observed in trauma patients. This might partially depend on the production of reactive oxygen metabolites[22]. Separation of the adherent cell fraction (macrophages) also restored peripheral blood NKCCs in bile-duct-legated rats[27].

Some of the artificial materials are also able to decrease IL-2 receptor density on NK cells *in vivo* [37]. Since IL-2 strongly stimulate NKCCs, this leads to their diminished activity.

The results obtained in this study indicate that shortly after surgical stress the polyester implant transiently modulates the immune system and influences peripheral blood leukocytes. Therefore, it is possible that an implanted prosthesis prolongs surgical stress or that the macrophages activated by the implanted prosthesis produce cytokines that influence the HPA axis and the release of hormones that affect circulating immune cells. Other factors that might affect NKCCs include a decrease in the density of IL-2 receptors on the immune cells.

To clarify which mechanism is more important, further investigations are needed. Since NK cells are also significant in the control of viral infections and carcinogenesis in humans, our data suggest that patients with polyester prostheses are probably exposed to a higher risk of viral infection and/or metastases due to side effects of the implant, and not only to surgical stress.

ACKNOWLEDGMENT

We thank Prof. Abdel Hakim Labidi, Ph.D., for helpful comments with this manuscript.

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