THE "SECOND SET" METHOD FOR ASSESSMENT OF IMMUNOGENICITY OF HEART VALVES

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The degree of immunogenicity of biological replacement heart valves depends essentially on subsequent preservation of their morphological structure. An indirect assessment of the immunogenicity of xenogeneic valves after different kinds of treatment can be made by the classical "second set" method on inbred animals. The choice of method is not accidental: Cellular phenomena of transplantation immunity are more precise indicators of the recipient's response to the graft than serum phenomena, and experiments on inbred animals enable general immunologic activity to be taken into consideration and cross effects to be excluded.

The object of this investigation was to determine the degree of reduction of immunogenicity of aortic valve complexes (AVC) subjected to combined chemical and enzymic treatment, followed by tanning in glutaraldehyde (GA) solutions and to compare the effectiveness of this treatment with the widely used Hancock's method, namely tanning in 0.5% GA solution. The method of combined enzyme and chemical treatment of hog AVC consisted of modification of the AVC with the enzyme terrilytin, which destroys cells, extraction of proteolysis products by salt solutions, followed by tanning of the AVC in solutions of GA (0.2-0.5%) for one month [1].

EXPERIMENTAL METHOD

Experiments were carried out on 50 inbred female WAG and August rats, for which interlinear transplantations are regarded as strongly allogeneic [2].

The recipients (WAG) were sensitized by subcutaneous transplantation of AVC from August rats into the region of the posterior surface of the neck. On the 28th day a specific donor's skin graft 1 × 1 cm^2 in area, freed from the subcutaneous fatty layer, was transplanted into the recipient in the lumbosacral region. The graft was sutured in place of a previously formed skin defect by means of an atraumatic needle. **The state of the skin grafts was assessed macroscopically,** noting the color, presence or absence of edema, turgor, and growth of the hair cover. The state of function of the newly formed vascular anastomoses in the skin graft was monitored by injections of 1-2 ml of a 10% indigocarmine solution into the posterior cutaneous vein of the thigh. If anastomoses were present the graft stained blue quickly and evenly during injection of the dye. Slowing or cessation of the blue staining of the graft was interpreted as the beginning of rejection. Sensitization of the 0 degree, developing after transplantation of AVC treated in various ways also was judged on the results of morphological investigation of the skin grafts, spleen, and regional lymph nodes between 5 and 8 days after the operation, and according to the results of the study of AVS 35 and 70 days after implantation. Histological sections were stained with hematoxylin and eosin, with picofuchsine by Van Gieson's method, by the PAS reaction, and by Brachet's method.

EXPERIMENTAL RESULTS

The dynamics of survival of the skin allografts is illustrated in Fig. 1.

After primary transplantation of an allogeneic skin graft the length of survival was 6.58 ± 0.35 days. Histologically, on the 8th day after transplantation, a lymphoid barrier was observed to have been formed at the host—graft boundary. Cells of the transplanted skin

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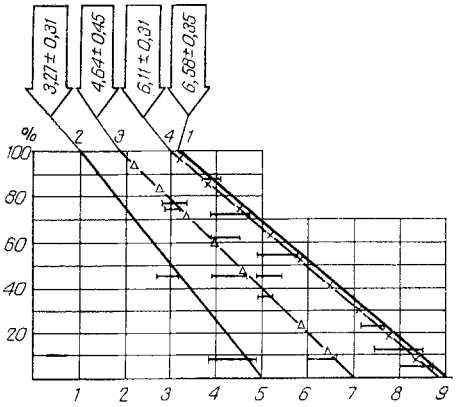

Fig. 1. Dynamics of survival of skin autografts in WAG rats after sensitization by heart valves treated in various ways. Abscissa, time of observation (in days); ordinate, survival of grafts (in percent). 1) 1st set; 2-4) 2nd set; 2) native valve, 3) native valve + GA (Hancock), 4) valve treated with enzyme + GA.

were partially reduced and the surface areas of the graft were infiltrated by neutrophilic leukocytes undergoing destruction. Collagen fibers of the allodermis appeared greatly swollen and edematous. These changes are evidence of the commencing rejection of the skin graft. Immune conflict was confirmed by changes in the lymph nodes, the cortex of which contained greatly hypertrophied follicles with lymphoid and plasma cells, whereas the medulla of the nodes was "stuffed" with these cells. Similar changes also were observed in the spleen.

In animals sensitized with native, untreated AVC rejection of the skin grafts began after 3.27 ± 0.31 days. Macroscopic signs of rejection were exhibited much sooner in this case, and for that reason material for investigation was taken on the 5th day after the operation. By this time the morphological picture of the allogeneic skin corresponded to the description given above (8th day). The lymph nodes and spleen were enlarged on account of hypertrophy of the follicles. Considerable accumulation of lymphocytes and, in particular, of plasma cells was observed in the lymph nodes. The latter totally filled the whole of the medulla of the lymph nodes and also the space between the follicles (Fig. 2a). In the implanted AVC by the 35th day fibrous structures of the aorta and the fibrous ring (mainly of elastic type) were relatively well preserved and twisted, and cells with elongated nuclei, evidently of connective-tissue origin, were identified between them. In the capsule surrounding AVC moderate infiltration by lymphocytes and histiocytes was observed (Fig. 2b).

In the series with preliminary sensitization of AVC, treated with the enzyme (removal of up to 100% of the cells in the cusp, just as in hog AVC), and tanned in GA, the length of survival of allogeneic skin was 6.11 ± 0.41 days. In most animals the cells of the epidermis were well preserved histologically (Fig. 2c). Homogenization and swelling of the collagen fibers of the allodermis observed in the previous series were found only in the regions of the sutures. The granulation tissue was more abundant and macrophages and fibroblasts predominated. Individual lymphocytes were present in the lumen of the well developed vessels. Hypertrophy of follicles and plasmacytosis were observed in the lymph nodes and spleen of some animals, but in others these phenomena were absent. Elements of implanted AVC were resorbed by macrophages and giant cells by the 35th day and the lymphocytic response was negative. The collagen fibers of the cusp remained fuchsinophilic and were gradually resorbed by macrophages and giant cells; by the 35th day the aorta had a more "inert" appearance with signs of resorption at the periphery (Fig. 2d). Of the structures of AVC 70 days after implantation only small fragments remained, surrounded by adipose tissue or a very thin capsule, with giant cells clustered around them. Where there were no giant cells fragments of aorta with loosely wound, twisted elastic fibers were seen.

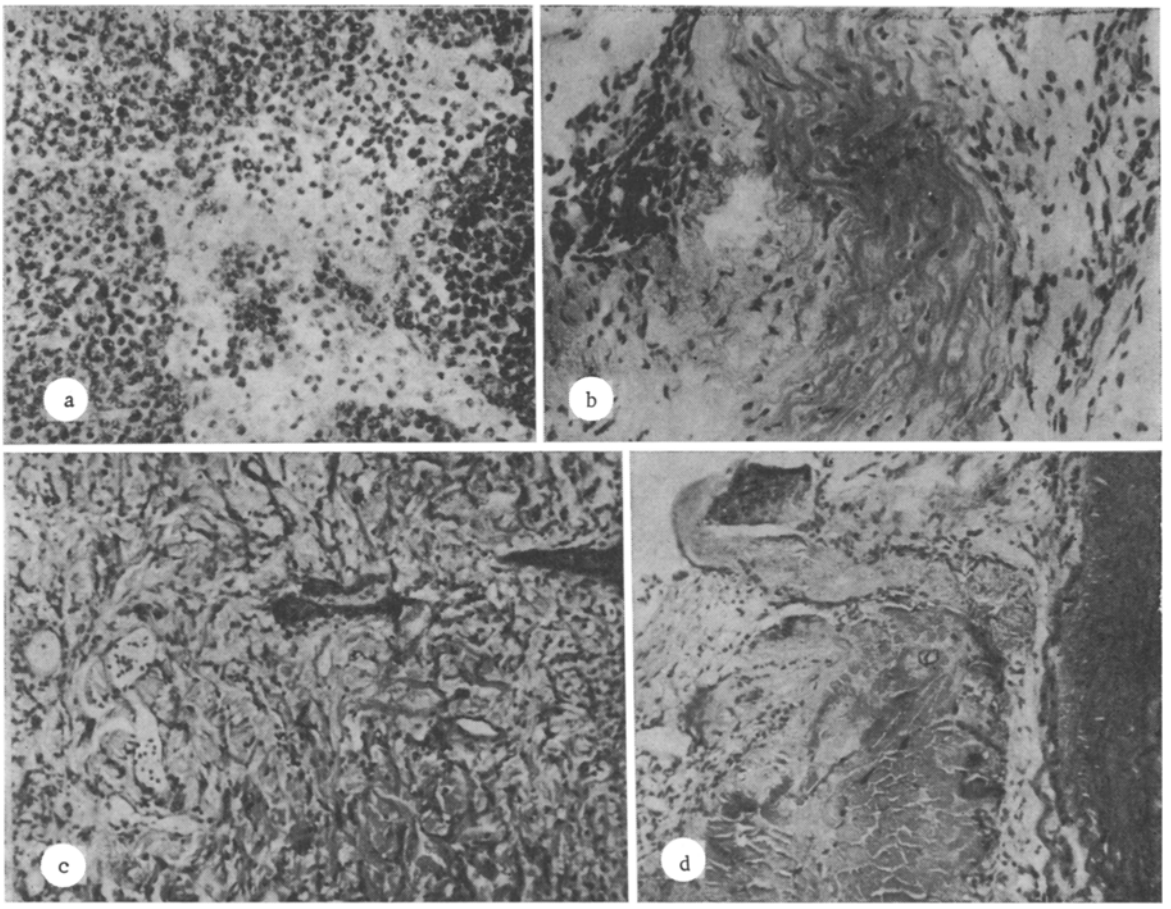

Fig. 2. Tissue response to native (a, b) and enzyme-treated (c, d) AVC. a) Hyperplasia of lymph nodes with marked plasmacytosis, 250×; b) structures of root of aorta on 35th day after implantation: moderate infiltration by histiocytes and lymphocytes, 250×; c) allogeneic skin on 8th day after transplantation, with sensitization by enzyme-treated AVC, 100×; d) resorption of cusp of AVC by macrophages and giant cells, aorta relatively well preserved on 35th day after implantation, 100×. Staining with picrofuchsine by Van Gieson's method.

In the series with preliminary sensitization of AVC treated with 0.5% GA solution alone, the grafts were rejected after 4.64 × 0.45 days, i.e., much sooner than in the series with enzyme-treated AVC. Morphological investigation showed that on the 5th day after skin grafting granulation tissue cells (macrophages, fibroblasts, endotheliocytes) could be identified subepithelially, the number of capillaries was relatively small, and the granulations were indolent in character. Around individual vessels slight lymphohistiocytic infiltration was observed, but there were significantly fewer lymphocytes than in the series with sensitization by native AVC. Hypertrophy of the follicles was observed in the lymph nodes, with local concentrations of plasma cells and lymphocytes in the medulla, in the region of intermediate and terminal sinuses. The structural elements of AVC on the 35th day showed various degenerative changes. The collagen and elastic fibers of the aorta were condensed in some areas, fused together, and partially hyalinized. The cusps of AVC were unevenly reduced in thickness and largely resorbed by macrophages and giant cells. In the peripheral zones of AVC, on the boundary with the capulse, lymphocytes and plasma cells were present (Fig. 3a). On the 70th day, in the region of the fibrous ring and elastic fibers of the aorta, deposition of calcium salts and lymphohistiocytic infiltration around fragments of the graft were observed (Fig. 3b).

Analysis of the dynamics of rejection of the skin grafts in all series of experiments, and the results of statistical analysis of the data gave the following results. On sensitization of WAG rats with August valves subjected to combined enzyme and chemical treatment, the specific donors' skin grafts were rejected after 6.11 ± 0.41 days, which did not differ significantly from the times of rejection of primary skin grafts (6.58 ± 0.35 days; $P > 0.4$). Next, after preliminary sensitization with enzyme-treated AVC the skin grafts were rejected later

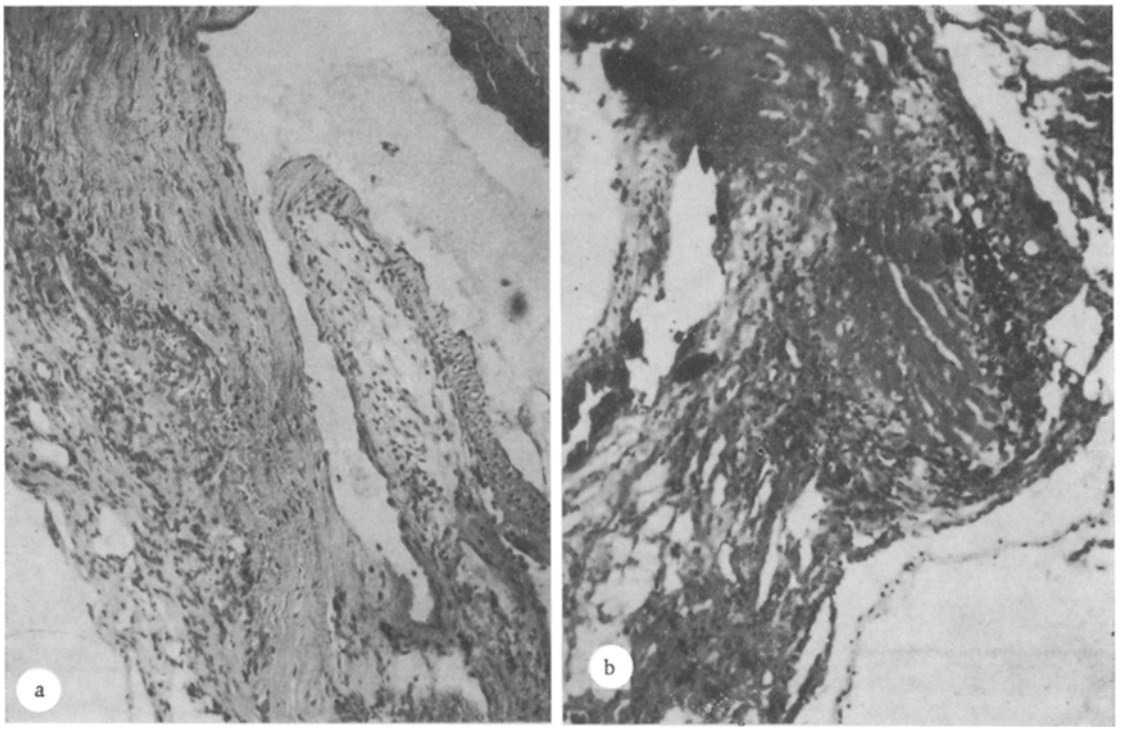

Fig. 3. Degenerative changes in AVC treated by Hancock's method. a) Regions of hyalinosis of aortic sinus and active resorption of cusp on 35th day after implantation, 100×; b) calcinosis and lymphohistiocytic infiltration of graft fragments on 70th day, 100×. Stained by Van Gieson's method.

than in the series in which they were sensitized by valves tanned in GA only ($P < 0.02$). The times of rejection of the skin grafts were 6.58 ± 0.41 and 4.64 ± 0.45 days respectively. Morphological study of the experimental material showed that signs of sensitization were most marked after transplantation of native, untreated AVC. After transplantation of AVC treated with GA along the intensity of lymphoid and plasma-cell infiltration of the skin was less than in the previous series, and in the spleen and lymph nodes the changes were similar in character. In this series, 70 days after implantation, the structures of AVC showed calcification, which was not observed in the other series. This fact is particularly interesting in connection with data in the literature on the development of similar processes in functioning biological valves of Hancock type [3, 4]. The mechanisms of this phenomenon have not yet been finally explained. However, on the basis of analysis of data in the literature and the results of our own investigations it can be postulated that deposition of calcium salts in the cellular and fibrous structures of the tissue of biological replacement heart valves is a sign of immune injury and the result of inadequate treatment of the biological tissue.

The investigation thus showed that the second set method, used on inbred animals (rats), can give a reliable estimate of changes in the immunogenicity of heart valves after treatment in various ways. Enzyme treatment, given before the stage of tanning of the valves in GA solutions, considerably reduces their immunogeneicity by comparison with that found after treatment by tanning in 0.5% GA solution alone.

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