MODELS IN ORTHOTOPIC CANINE CARDIAC ALLOTRANSPLANTATION

PROEFSCHRIFT

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CONTENTS

CHAPTER ONE

1.1. Introduction

Since human orthotopic allotransplantation of the heart became a reality in 1968, clinical experience has consolidated the knowledge obtained experimentally in the previous period. According to some investigators nothing more than this has been achieved. In an early comment on clinical heart transplantation, Dempster et al. (43) declared "Nothing new was proved by the incursion into human heart transplantation". The same authors continued "None-the-less experimental work must continue in the quite relentless way it has in the past".

Experimental orthotopic heart transplantation.

Many essential steps were clarified by experimental work before the first human allotransplantation of the heart was performed: the development of an adequate surgical technique for orthotopic heart transplantation (78), the demonstration of near normal function of the transplant despite denervation (35,44,45,79), and the clinical diagnosis and histopathology of acute transplant rejection (68,76,77,80). Acute rejection after one week is the ultimate fate of the cardiac allograft if donor and recipient are unrelated and no immunosuppressive drugs are given to the recipient.
Also a limited experience was gained concerning the effectiveness of immunosuppressive drugs on the survival of the heart transplant, and it was demonstrated that recovery from acute rejection was possible by the timely use of such therapy. Small areas of scar tissue marked the location of tissue destruction that had occurred due to the damage of the capillaries venules and myocytolysis. These scar tissue formations occur because the parenchymal cells of the heart are unable to regenerate (47, 68).
Lower et al. (79,81) described the longest survival of 18 months for a heart transplant in a dog given immunosuppressive therapy in the form of azathioprine and methylprednisolone. These workers (81) however expres-

sed their concern about the microscopic findings of the vascular lesions which they observed in the transplant. They wrote "Such arterial lesions appear to represent a form of chronic rejection which may in the early stages go undetected by the electrocardiogram". This and other experience (13,22,23,31,55,67.) with the use of immunosuppressive drugs in dogs and calves after orthotopic allotransplantation of the heart, did not give much hope for prolonged survival following clinical heart transplantation.

Clinical orthotopic heart transplantation.

The anticipation of better results in human heart transplantation was based on the previous experience and improvement in clinical renal transplantation (6). Also, the protocols for immunosuppressive treatment were mainly based on the experience obtained in human cadaver renal transplantation (6, 37).
In December 1967, eighteen days after a heart transplant in Cape Town (5) the first cardiac transplant patient died. In 1968 and in the following years the world was confronted with a wave of clinical heart transplants, the results of wich sometimes gave rise to severe criticism (43).
The results obtained in experimental work before 1968 could now be confirmed in clinical practice. For example, the surgical technique of Lower et al. (78), with a single modification (4, 38), was used by all surgical teams and turned out not to be a major cause of hospital mortality.
The essential element of Lower's technique is to excise the heart of the recipient by a midatrial incision and to use these atrial cuffs for the venous anastomoses. This avoids the need of making multiple venous anastomoses of both caval veins to the right atrium and the separate lung veins to the left atrium.
Barnard and Cooley (4, 38) modified the technique by dividing and ligating the superior caval vein of the donor heart. The right atrium of the donor heart was opened through an incision starting at the inferior caval vein up into the atrial wall away from the area of the sinus node and its arterial supply. This modification minimized the risk of direct injury to the sinus node.
When prophylactic immunosuppressive therapy prevented the onset of the rejection process, adequate function of the heart transplant was achieved both subjectively and objectively (8, 50, 54, 106, 109.). The experimentally acquired criteria for the diagnosis and the characteristic histopathology of acute rejection were clinically confirmed.

Just as in experimental animals, the acute rejection process was the major cause of the *early death* in patients after heart transplantation. In spite of intensive immunosuppressive therapy, this process turned out to be largely irreversible. Besides, the high dosage of immunosuppressive drugs was often the cause of a fatal intercurrent infection (24, 100).

Initially the *long term results* in patients after heart transplantation, were comparable to the results of previous experimental work. The first published results clearly demonstrated this. By the end of 1968, all over the world, 65 transplants had been registered. At the time of the report in October of that year, 33 patients had already died (53). Even at the Stanford University Clinic in Palo Alto, where Shumway and his co-workers had applied themselves with utmost care to the experimental and clinical problems of heart transplantation, there was a mortality of 78% in the first year following transplantation (50). For one surgeon this was enough then to state "I reserve the right to change tomorrow, but I am proud of our restraint in not performing heart transplantations yesterday" (57).
However disappointing these initial results, they were better than what could have been expected on the basis of previous experimental work.
In spite of this high early mortality in the first year after transplantation the Palo Alto group achieved a one year survival rate of 22% in their patients (50). A similar percentage of one year survival had never been achieved in any experimental research. In the following years, it proved possible to improve the results to the same level of success as in clinical renal transplantation with unrelated donors. For example, in 60 patients who underwent heart transplantation since the end of 1973, the survival at one, two and three years was 66, 63 and 58% respectively (7).
A number of factors have played a role here (49). This included an increased experience in the use of immunosuppressive drugs and the verification of the effect of this therapy on acute rejection by means of transvenous myocardial biopsy (26, 27, 28). Also, anticoagulant therapy and a low cholesterol diet with strict caloric limitation was claimed to be used with some success against the process of chronic rejection (77).
Sofar, human heart transplantation has provided no firm data concerning the significance of tissue typing in the outcome of these transplants with inevitably unrelated donors (108).
The results from research in clinical renal transplantation (93) and from experimental work indicate that previous blood transfusions donated to the recipient can have a favourable influence on the kidney transplant

prognosis. The extent, to which blood transfusions have influenced the results of heart transplantation is uncertain, but this has been mentioned in literature as a possible relevant factor (39, 58).

Chronic cardiac allograft rejection was the next major obstacle in clinical transplantation and limited to a great extent the possibility of achieving a considerable prolongation of life after transplantation (48, 51, 116). The progressive course of this type of rejection poses great diagnostic problems. This type of rejection results in a syndrome of severe coronary artery disease which, for most patients, was identical to the original disease necessitating heart transplantation.

After ten years of clinical heart transplantation, only 77 (23%) out of 338 patients, operated upon in this period are still alive (107). These figures clearly demonstrate that acute and chronic rejection have remained at least partially unsolved problems. A continuation of experimental work was therefore necessary, even during the clinical phase of heart transplantation.

The continuation of experimental orthotopic heart transplantation.

In his thesis Jongsma (63) reviewed the results of orthotopic heart transplantation in various laboratories. Although most of the investigators used dogs, others used pigs, calves or primates. The surgery was carried out with the help of extra corporeal circulation in larger experimental animals and with deep hypothermia in smaller animals (20, 21, 31, 42, 65, 66.). It is not surprising that for many research workers the management of the surgical technique was of first priority as many attempts at orthotopic transplantation resulted in a too high operative mortality (56, 71, 77, 87, 88, 89, 105, 118.). The fact that very few laboratories were provided with an orthotopic heart transplant model for further research, contributed to this lack of success.

It is easy to understand that most of the experimental work during the phase of human heart transplantation was clinically oriented. The clinician was confronted with the actual problems of the cardiac transplant patient. Methods had to be developed for early and accurate diagnosis of rejection, and for the optimal treatment with the available immunosuppressive drugs.

The clinical and histopathological features of long term survivors after heart transplantation had to be defined. Under pressure of these clinical questions, there was limited progress in fundamental research, oriented towards the basic immunological problems of heart transplantation. In practice this meant that there was little opportunity for immunologists to study specific questions in a model of orthotopic heart transplantation in experimental animals.
Therefore, there is a clear need for the clinical surgeon, who is able to perform orthotopic heart transplantation in experimental animals, to work together with immunologists to develop their own models for fundamental research into transplantation. Such a cooperation between the surgeon and the immunologist in the study of heart transplantation forms the basis of the experiments in heart transplantation that will be described in the following chapters.

1.2. Rationale of the study

The preference for the canine orthotopic heart transplant model used in this study, is based on the work of Jongsma (63), who compared the orthotopic with the heterotopic model of heart transplantation. Jongsma concluded that the impaired coronary circulation in the heterotopic position caused extra ischemic damage to the graft and resulted in a specific rejection process characterized by the predominance of necrotizing arteritis and infarction necrosis.

Model of acute rejection.

In 1961 Lower was the first to publish a reproducable surgical technique of orthotopic heart transplantation in dogs. Using unrelated donor-recipient combinations and without the use of immunosuppressive drugs, he provided a classical model for acute rejection of an orthotopic heart transplant in an experimental animal. Twenty dogs died from acute rejection with a mean survival time of one week and a range of 4 to 21 days. This was recognized as a great achievement by everyone familiar with the history of heart transplantation. This history of heart transplantation has been reviewed by Hairston (52) and since then by many other investigators.

Jongsma (63) confirmed these observations under the same conditions in 13 dogs after orthotopic cardiac allografting. He achieved a mean survival time of 9.6 days with a range of 5 to 15 days. The microscopic features confirmed the classical picture of acute allograft rejection. This histological picture was characterized by:

a) an infiltration of mononuclear cells
b) rupture of capillaries and venules
c) myocytolysis of myocardial fibres and eventually
d) a necrotizing arteritis of small arteries.

Thus, fundamental research was provided with a classical model of acute rejection of an orthotopic canine cardiac allograft.
However, the importance of tissue typing in unrelated donor-recipient combinations still remains to be investigated in this preparation. Neither has the effect of the transfusion of homologous or so called third party blood, been examined in this model of orthotopic heart transplantation.

Model of chronic rejection.

The results obtained after attempts to construct a model for a slowly developing rejection process, or chronic rejection of an orthotopic canine allograft are less clear and very difficult to delineate.

A *clinical approach* is usually described in the litterature to achieve a prolongation of graft survival in experimental animals. To accomplish this goal, drugs like corticosteroids, azathioprine, methotrexate and anti-lymphocytic globulin are given to the recipients. Additional dosages of these drugs are given at times of an apparent acute rejection episode (13, 22, 23, 31, 67, 79). This clinical approach results in only a small number of long term survivors, especially when one considers the large numbers of experiments that have been performed. In addition, the results are not easily reproducible and the non-specific suppression of the immune response of the host by these drugs causes a reduction in resistance to infection. A valid interpretation of the results is also made more difficult in most cases by the administration of a high dose of immunosuppressive drugs which very often results in a complicated picture of drug intoxication.

To elucidate a *systematic approach* in the use of immunosuppressive drugs, the work Jongsma reported should be mentioned (63). He published a systematic use of immunosuppressive drugs in 13 dogs after orthotopic heart transplantation with unrelated donors.
Drugs were given according to the following schedule:
Initially high doses of methyl prednison (10mg/kg bodyweight) and azathioprine (10mg/kg bodyweight) were given, starting at surgery and on the first postoperative day respectively. The dosage was tapered off during the following days until a daily maintenance dose of azathioprine of 3 mg/kg bodyweight and prednison of 1 mg/kg bodyweight. On the 7 th postoperative day the whole schedule was repeated and again gradually diminished to the maintenance dose. From that day on (the 12th day), regardless the occurrence of an acute rejection process, the maintenance dose was continued to the end of the experiment. This fixed schedule of immunosuppression resulted in:

a) a wide range of survival time of the cardiac allografts, from 7 to 282 days with a mean survival time of 64 days;
b) the occurrence of a large number of infections, 4 out of 13 animals;
c) the occurrence of drug intoxication in 9 out of 13 experimental animals.

In summary, neither the clinical use nor the systematic use of immunosuppressive drugs in orthotopic heart transplantation in unrelated dogs had lead to a reproducible and predictable long term survival. The frequent occurrence of infections and drug intoxication in the experimental animals hampered a proper judgement and a mutual comparison of the results. As a result of these unpredictable factors it is unlikely that orthotopic heart transplantation in unrelated dogs followed by the use of presently available immunosuppressive drugs, will result in a suitable basic model for chronic rejection.

Again it should be stated that the influence of tissue typing and the administration of homologous blood transfusions during transplant surgery or thereafter on graft prognosis was not taken into account in any of these investigations on long term survival.

From this discussion on the development of a model for chronic rejection of the orthotopic canine cardiac allograft, the emerging alternatives are:

1) To prolong graft survival without the detrimental effects on the recipient by the use of immunosuppressive drugs. As will be demonstrated, DLA matching served this purpose in the present study. In the laboratory for experimental surgery of the Erasmus University in Rotterdam, much work has been done demonstrating the existence in dogs of a main histocompatibility complex, the DLA complex. It is analogous to the human HLA complex. For an increasing number of organs, prospective tissue typing for this DLA complex proved to be of importance for graft survival in beagle siblings. Differences in graft survival times were even demonstrated without the use of immunosuppressive drugs (122).

2. To develop a surgical technique of orthotopic cardiac allografting in dogs without the need for homologous donorblood.
In the preceding pages it was stated that all clinical and experimental investigation on orthotopic heart transplantation included the use of homologous donor blood. In the past the reason has been that surgery of this kind necessitated blood transfusions because of the use of extracorporeal circulation, heparinization of the recipient and the postoperative blood loss.
In contrast to this, great restraint has been exercised in kidney transplantation in the use of pretransplant blood transfusions in order to minimize the risk of an immunization of the recipients against donor antigens. This transfusion policy in clinical as well in experimental kidney transplantation is at present widely debated (90, 103). Clinical and experimental results are now available which show a benificial effect of prior blood transfusions on kidney graft survival.
In clinical heart transplantation Coulson et al. (39) reported that patients who had previous heart surgery on cardio pulmonary bypass appeared to fare better than those who did not have that type of surgery in the past. Further discussion on this aspect of the use of homologous donor blood is presented in the last chapter of this thesis.
Thus far, no information has been reported on the effect of homologous donor blood on graft survival in orthotopic canine cardiac allografting.

1.3. Objectives of the present study

In accordance with the two alternatives discussed in the preceding pages the

aim of the study was twofold and dictated the design of the first and second experiment.

First objective and first experiment:

The first objective was to develop a model of chronic rejection of an orthotopic canine cardiac allograft using DLA-identical beagle littermates without immunosuppressive drugs. Thus, the detrimental effects of immunosuppressive drugs were to be specifically excluded.

Second objective and second experiment:

The second objective was to design and test a surgical technique of orthotopic canine cardiac transplantation without the need for homologous donor blood.

It should be noted that at the time the first experiment was performed, it was believed impossible to meet the second objective with the current state of surgical technique. Therefore the results of the second experiment could not be included as a prerequisite in formulating the first objective.

CHAPTER TWO

MATERIAL AND METHODS

2.1. Animals.

In the *first experiment* prospectively tissue typed adult littermate beagles from an outbred colony were used as heart donor and recipient.
The beagles were obtained from "the Centraal Proefdieren Bedrijf T.N.O." (Zeist, the Netherlands, Dr. J. van Vliet) and from our own colony.
The weights of the recipients ranged from 9.4 kg to 19.5 kg (mean 13.6 kg).
Fresh heparinized blood for pump-priming and citrated blood for post-operative blood transfusion were collected from mongrel dogs.
In the *second experiment* both recipient and donor dogs were mongrels, unrelated to each other and matched only for size and weight. The mean body weight of the 10 successfully transplanted mongrels was 11.5 kilogram.
In accordance with the aim of this experiment, no homologous third party donor blood was used, neither for pump-priming nor for postoperative bloodtransfusion.

2.2. Tissue typing.

Prospective tissue typing was performed in the first experiment in which littermates were used as heart donor and recipient.
The microcytotoxicity test of Kissmeyer-Nielsen, modified by Van Rood et al. (101), was applied to test dog peripheral lymphocytes. The production and evaluation of dog allolymphocytotoxins for donor selection have been reported by Vriesendorp et al. in more detail elsewhere (119, 121).

2.3. Design of the first experiment.

Based on identity or nonidentity for the serological defined (SD) antigens

two groups of beagle littermate pairs were formed for this first experiment.
In *group one* heart donor and recipient were DLA-identical beagle littermates.
In *group two* heart donor and recipient were DLA-nonidentical beagle littermates and served as a control.
None of the recipients from these two experimental groups received any specific type of immunosuppressive treatment before, during, or at any time after orthotopic cardiac transplantation.

2.4. Surgical procedure.

The surgical technique for orthotopic cardiac allografting was originally described by Lower et al. (78) and has been reviewed in great detail by Caves and Dong (25). This surgical technique was adopted for this study as the method of choice. Our surgical procedure and the use of a Dreissen roller pump with a Q 130 Temptrol disposable bubble oxygenator has been reported by Jongsma (63).
In the first experiment, total pump-priming consisted of fresh heparinized blood and postoperative blood transfusions were given with fresh ACD blood. The blood was collected from mongrels just prior to surgery.
For the second experiment homologous third party donor blood was not used.
Instead the priming volume of the pump consisted of 400 ml HAEMACCEL® (Behring) only. At the conclusion of extra corporeal support the total priming volume of the pump was returned to the recipient.
The setup of the autotransfusion system after completion of orthotopic cardiac transplantation is illustrated in Fig. 1. Before closure of the chest an infusion line for continuous acid-citrate-dextrose 16% (ACD) drip was positioned inside the pleural space where the citrate mixed with the blood still leaking in the pleural cavities.
This ACD blood was collected from a single thick chestdrain by intermittent manual suction using a syringe. The collected blood was then centrifugated.
After discarding the hemolytic plasma, the resulting packed cells were diluted with saline and returned to the animal by intravenous infusion.
Depending on the amount of citrate used, this was counteracted by intravenous infusion of calciumchloride. The hemolytic plasma loss was substituted with haemaccel.

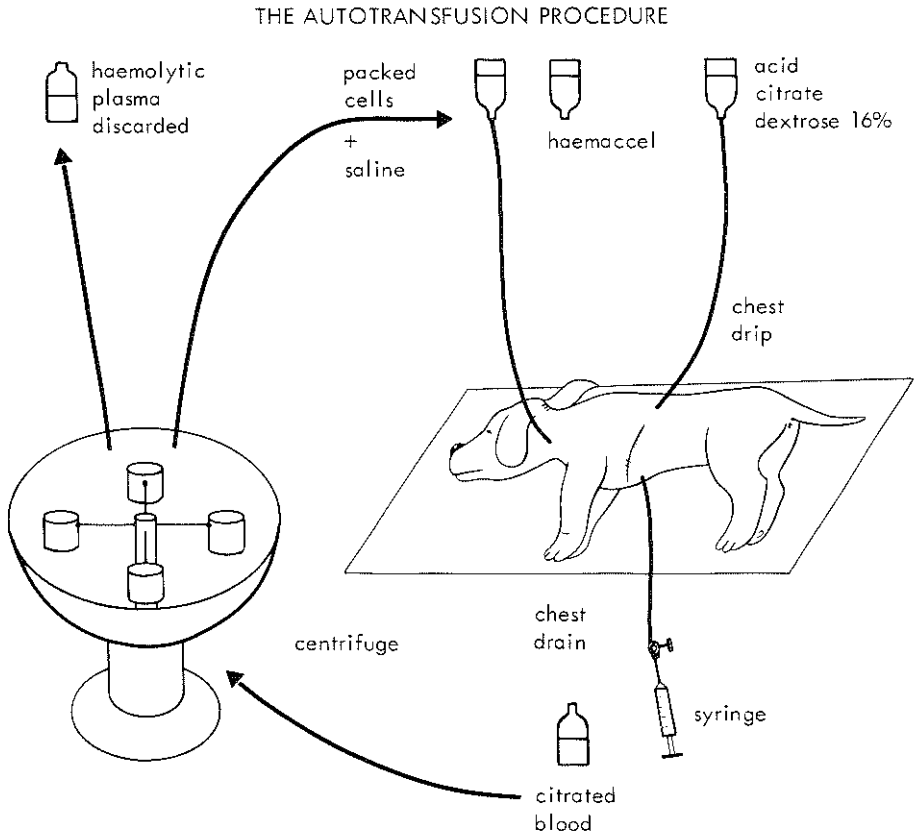


Fig. 1: The postoperative setup of the autotransfusion system after orthotopic canine cardiac transplantation.

2.5. Postoperative management.

Approximately 6 hours after surgery the dogs were fully awake and active and were returned to their cages.
On the first postoperative day the animals were allowed unrestricted fluid intake. From the second day on they also were fed solid food.
Antibiotics were given intramuscularly: on day 0,3 doses of 1 million units penicillin and 0.5 g streptomycin; on days 1 to 6, 2 doses of 1 million units penicillin and 0.5 g streptomycin daily.

2.6. Exclusions.

All dogs, failing to survive the surgical procedure or immediate postoperative period after transplantation or those dogs ultimately dying for reasons other than rejection as a major cause, were excluded from this study. Their causes of death are discussed in more detail in the next chapter.

2.7. Survival time.

In orthotopic cardiac transplantation graft survival time coincides with the survival time of the animal.
Four dogs from the first experiment were sacrificed by an overdose of Penthotal because these dogs finally manifested a severe end-stage of the rejection process and were unable to support or to feed themselves.

2.8. Electrocardiographic studies.

Daily electrocardiographic limb lead tracings were taken on each dog after transplantation.
A Hewlett Packed ECG recorder no. 1511 A (monochannel), recorder speed of 25 mm/sec. (voltage 1 mV = 10 mm), was used throughout this study and calibrations were made prior to recording.
Because the dogs mediastinum is a loose structure, special attention was paid in keeping each animal in a right lateral recumbent position during ECG recording.
The initial postoperative electrocardiogram was considered a base-line standard in each recipient. Peak-to-peak QRS voltage in lead II and cardiac rhythm were determined.

2.9. Histopathology.

At the time of recipient death, the thoracic and abdominal contents were examined grossly.
Fresh representative sections of the graft were preserved in 4 per cent phosphate buffered formalin (pH 7.0) and imbedded in a paraffin-paraplast beewax mixture. Sections were cut 5 to 7 micron thick and stained

with hematoxilin-azophloxin-saffron (HAS) for subsequent histologic examination.
The microscopic findings of graft rejection were graded from 0 (minimal change) to 4 (extensive change) in a semi- quantitative manner.

2.10. Hematology.

Hematological changes were evaluated in the second experiment, following orthotopic canine cardiac allotransplantation, using total hemodilution and postoperative autotransfusion. Bloodsamples were taken to determine hemoglobin concentration, hematocrit, the number of platelets, the fibrinogen concentration in the plasma, and the appearance of fibrinogen degeneration products. The platelets were counted with the Coulter counter model C.
The fibrinogen concentration was measured according to Clauss (34).
To determine the presence of fibrinogen degeneration products the technique of Laurell (74) was employed.
These samples were collected before surgery, four hours after transplantation, and the first day, the second day and one week after transplantation.

CHAPTER THREE

RESULTS

3.1. First experiment.

A total of 31 orthotopic cardiac allotransplantations were performed among prospectively tissue typed beagle littermates. Cold ischaemic times for the grafts during transplant surgery ranged from 35 to 55 minutes. The amount of postoperative blood transfusioned ranged from 250 to 1200 ml.

Exclusions.

In total 15 animals were eventually excluded from the study.
Surgical mortality resulted in the exclusion for further analysis of 12 animals (39%).
Uncontrollable leakage from the suture line at the level of the friable supravalvular aortic tissue in the immediate postoperative period caused the highest early mortality rate of 29% (9 animals).
One animal died because of a technical failure in the management of extra corporeal circulation. As a result the dog became decerebrated and subsequently died.
Complete heartblock was the cause of death in two animals (6%) and both died on the second postoperative day. The two dogs were DLA-nonidentical to their heart donors. Postmortem histopathological examination of the grafts from the recipients did not reveal signs of rejection. The two beagles were excluded from the study.

Another two beagles, both DLA-identical to their littermate heart donor, died from bilateral pneumonia on the 7th and 13th postoperative day respectively. No clinical and histopathological signs of rejection were observed. Both animals were excluded from the study for further analysis.

Perforation of the aortic suture line caused sudden death in one animal on the tenth postoperative day. Donor and recipient were tissue typed as DLA-identical pair. Also in this case, postmortem histopathological examination of the graft, showed no signs of rejection and only an area of local tissue necrosis was seen at the point of the aortic perforation. This animal was also excluded from the study.

Survival times (Table 1).

A total of 16 dogs, 8 in group one and 8 in group two, recovered from transplant surgery, but all these animals later died from the rejection response as the major cause of death.

Table 1
First experiment. Orthotopic cardiac allografting of beagle to littermate.

No immunosuppression.

	GROUP ONE	GROUP TWO
DLA Typing:	Identical (8)	Non-identical (8)
Survival time, days:	247(S), 164(S), 132(S), 80, 47, 44, 38, 21	48, 44(S), 17, 14, 11, 11, 10, 9.
M.S.T., days:	96,6*	20,5

M.S.T. = mean survival time
( ) = number of animals
(S) = sacrificed
* $P < 0.01$ (two-sided Wilcoxon test).

Group one.

The survival times of 8 animals after orthotopic cardiac allotransplantation in this group of DLA-identical donor-recipient beagle littermate pairs were 247, 164, 132, 80, 47, 44, 38 and 21 days respectively.
The three longest surviving animals were sacrificed because, as has been stated in the previous chapter, they developed a severe end-stage of the rejection process and were unable to support or to feed themselves.
Mean survival time of the transplanted beagles in this group was 96.6 days, with a range of 21 to 247 days.

Group two.

In this group of 8 beagles after orthotopic cardiac allografting between DLA-nonidentical donor-recipient littermate pairs, the survival times were respectively 48, 44, 17, 14, 11, 11, 10 and 9 days.
Also in this group one animal, the second longest survivor, was sacrificed at day 44 because it developed a severe end-stage of the rejection process.
Mean survival time of the transplanted animals in this group was 20.5 days, with a range of 9 to 48 days.

The differences between the mean survival times observed in group one and group two are statistically significant ($P < 0.01$ in two-side-test. Wilcoxon test).

Clinical signs of rejection.

While in the first group 5 out of 8 animals developed ascites during the course of their protracted survival, only two long surviving dogs in group two demonstrated this clinical feature, which is illustrated in Fig. 2.
In the short term surviving dogs, dying of acute rejection, the first clinical signs observed were lethargia and anorexia.
If no signs of rejection were present, the dogs looked healthy and showed normal activities (Fig. 3).

Electrocardiography.

In the immediate post-transplant period after electrical or spontaneous

defibrillation, all hearts of the surviving dogs in group one and group two demonstrated normal sinus rhytm.
Occasionally arrhythmias were present during the first operative day and disappeared by the second or third day. These arrhythmias did not produce obvious alterations in the clinical status of the animals.
An example of arrhythmias in the early postoperative period is presented in Fig. 5A. This arrhythmia disappeared on the second postoperative day (Fig. 5B).

The occurrence of electrocardiographic abnormalities, reflects the pathological changes in the transplanted heart during the course of the rejection process.
Two changes which correlate well with the histological findings of acute rejection are a decrease in the QRS voltage (Fig. 4), seen best in the R wave of limb lead II, and the late occurrence of arrhythmias (80).
In both groups one and two, the time of first appearance and the nature of these electrocardiographic changes were recorded and also the time interval in days between first recording of the electrocardiographic abnormality and the time of death of the experimental animal (Table 2).

Table 2

First experiment. Recording of first appearance of electrocardiographic changes.

	No. of dogs	Decrease in QRS voltage	Arrhythmias	A.T.I. (days)
Group one:	8	3	5	38.3
Day of first appearance:		13, 20, 28	34, 62, 83, 93, 131	range: 9-115
Group two:	8	5	3	9.9
Day of first appearance:		4, 5, 5, 7, 9	9, 15, 24	range: 4- 36

A.T.I.: Average Time Interval in days between first recording of the electrocardiographic abnormality and the time of death of the experimental animal.

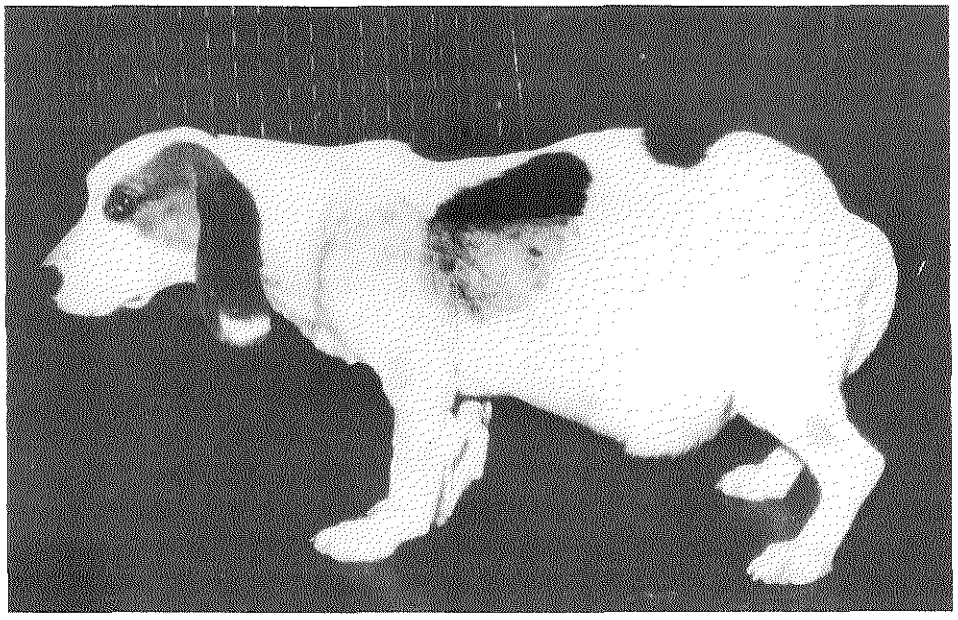


Fig. 2: Dog presenting ascitis 142 days after orthotopic cardiac allografting.

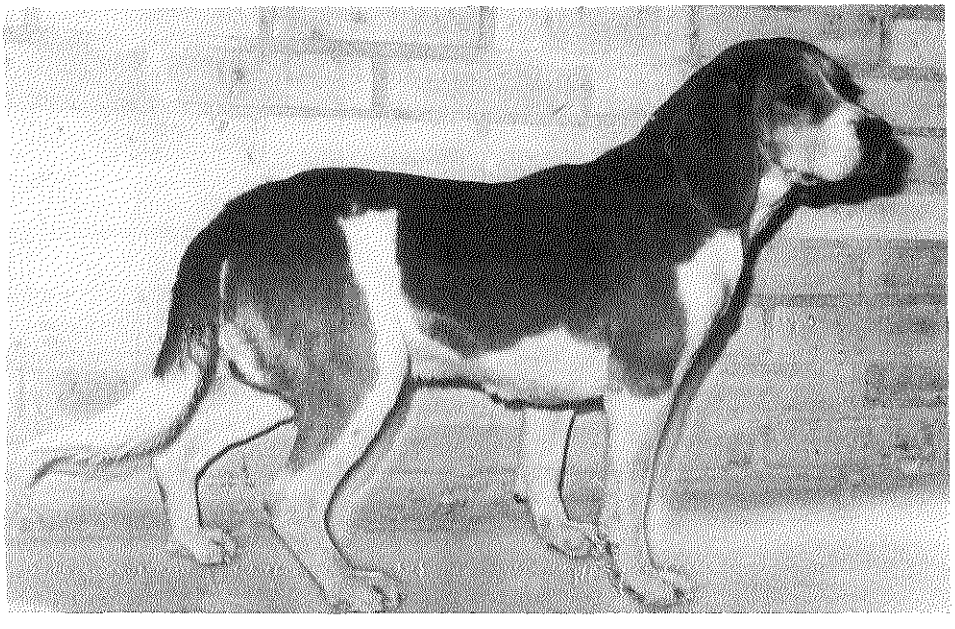


Fig. 3: Dog presenting no clinical signs of rejection eight weeks after orthotopic cardiac allotransplantation.

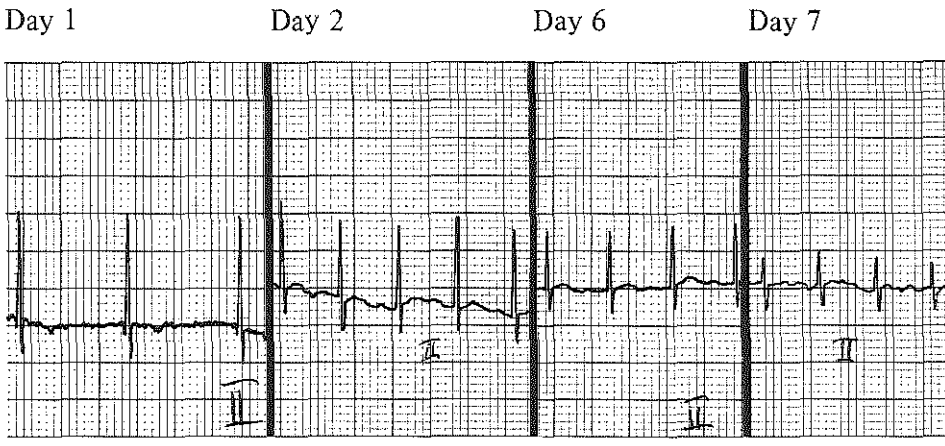
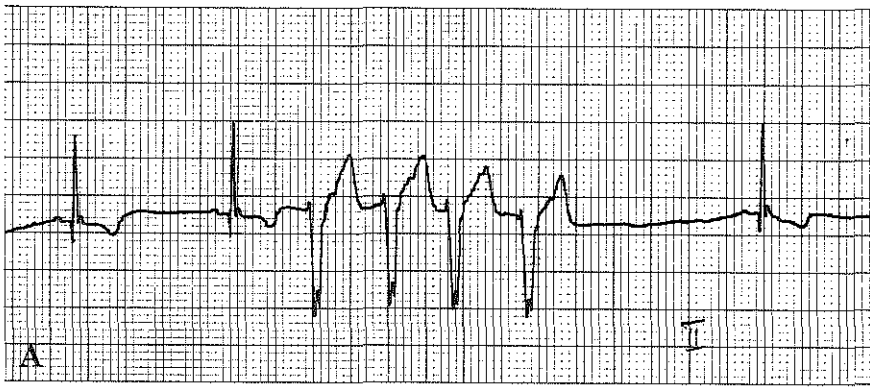


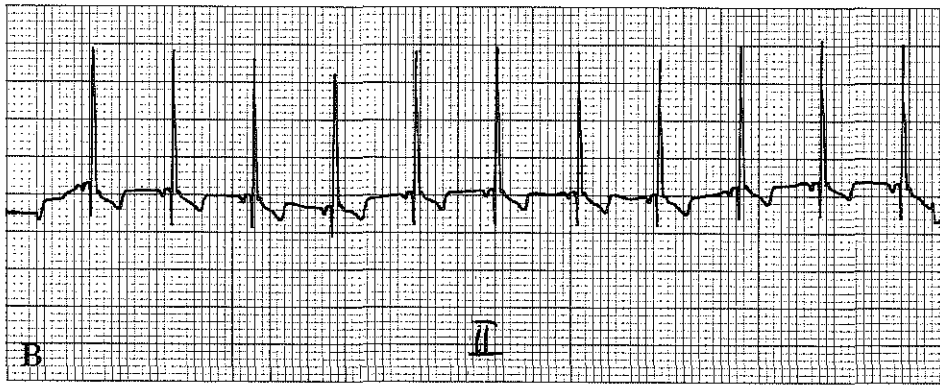
Fig. 4: Post-operative E.C.G. recording inacute rejection. Dog BD982.

In group one the daily ECG recordings of the three shortest surviving dogs, after orthotopic cardiac allotransplantation from a DLA-identical littermate donor, demonstrated a persistent decrease in the QRS voltage, starting at day 13, 20 and 28 respectively.
The ECG recording of the other 5 dogs showed arrhythmias as the first electrocardiographic abnormality which started at day 34, 62, 83, 93 and 131 respectively and persisted thereafter.
The average time interval between first recordings of the ECG changes and the times of death of the dogs in this first group was 38.3 days with a range of 9 to 115 days.
The first appearance of an arrhythmia in a long surviving dog is illustrated in Fig. 5C.

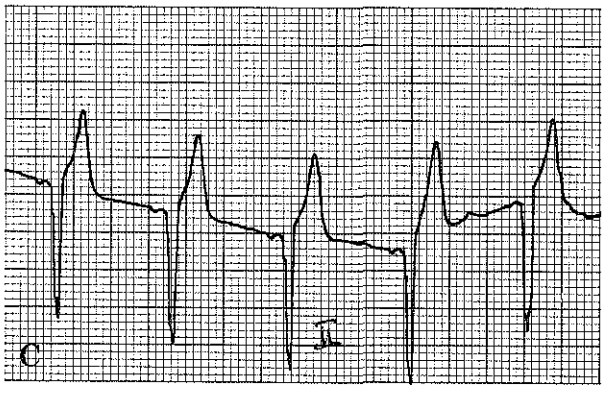
In group two a decrease in QRS voltage was recorded as the first electrocardiographic change in 5 dogs, starting respectively at day 4, 5, 5, 7 and 9. The three longest surviving dogs in this second group demonstrated arrhythmias as the first electrocardiographic abnormality on their daily ECG recording at respectively day 9, 15 and 24.
In this group of 8 beagles after cardiac allografting between DLA-nonidentical littermate pairs, the average time interval between first recordings of ECG changes and the times of death of the dogs amounted to 9.9 days, with a range of 4 to 36 days.



Day 1: Sinus rhythm. ventricular tachycardia.



Day 2: Supraventricular rhythm.



Day 93: Supraventricular rhythm. Intraventricular rhythm disturbances. Dog BD9173 late occurrence of arrhythmia during chronic rejection.

Fig. 5: Post-operative E.C.G. recordings.

Histopathology.

The general autopsy findings correlated well with the clinical course of the rejection process in both groups one and two.

At autopsy the clinical signs of left and right sided congestive heart failure, or a combination of both, as observed during the course of the rejection process, paralleled the findings of various amounts of pleural effusion and of free fluid in the abdomen. The amount of fluid was directly proportional to the duration of the rejection process and in the two extreme cases amounted to over 1300 ml of pleural effusion and more than 5000 ml of free fluid in the abdominal cavity. In one of these extreme cases the extremities of the animal showed severe edema.
The degree of congestion of the various organ systems appeared to be proportional to the severity of left and right sided heart failure, as reflected by the amount of pleural and abdominal effusion.

Graft findings.

To illustrate the characteristic histopathological differences in the transplants between group one and group two, the most typical findings in one representative specimen of each group are described.

Macroscopical features.

Group one (dog BD 9173, survival time 132 days).

The chest cavities contained a minimal amount of clear yellow fluid.
In the abdomen 5000 ml free fluid was present. There were dense epicardial adhesions to the left lung and the previously opened pericardium. The heart was enlarged and the walls of both ventricles were thickened.
The color of the myocardium was normal. There was no myocardial hemorrhage and edema typical of acute myocardial rejection. The epicardial surfaces showed only slight roughening and opacity.
The heart cavities, opened along the lines of bloodflow, were filled with post-mortem clots not adhering to the endocardium.
All suture lines were well healed and smooth.
The four cardiac valves were remarkably deformed. The cusps were slightly

retracted and the free edges of the valves were rolled, thickened, and irregular. Both atrioventricular valves showed evidence of shortening and distortion of the chordae tendiniae.

Group two (dog Bd 9118, survival time 10 days).

There was 250 ml of clear yellow fluid present in the chest cavities and a minimal amount of fluid was present in the abdomen.
There was evidence of a fibrinous pericarditis and an increased amount of fluid was present in the pericardial cavity. The heart was enlarged and the myocardium edematous.
After opening along the lines of bloodflow, the heart cavities were found to be filled with post-mortem clots not adhering to the endocardium. The myocardium was dusky red and felt rubbery.
On the endocardial side of the myocardium many small, ill defined foci of hemorrhage were present. All suture lines were smooth. The heart valves appeared to be slightly edematous.

Microscopical features.

Group one (dog Bd 9173, survival time 132 days).

Within the myocardium there were only slight focal infiltrations of histiocytes and lymphocytes compared to the dogs in the other group.
The small interstitial vessel walls were intact and the small arterioles diffusely demonstrated thickened, hyalinised walls.
The myocardium showed scattered areas of infarction necrosis, characterized by hyalinization of muscle fibres and a scarcity of cellular infiltrate.
The most profound lesions were observed in the intramural and epicardial coronary arteries. The lumina of the arteries were extremely narrowed or obliterated, mainly because of an intense proliferation of intimal cells.
Many intimal cells showed cytoplasmic vacuolization. The media and adventitia showed various amounts of fibrosis, often with total obliteration of muscle fibres. The infiltration of all layers of the vessel wall with lymphocytes, plasma cells, histiocytes and an occasional granulocyte is a prominent feature.
These changes, found to be typical for chronic rejection, will be indicated

as a *chronic obliterative arteritis* to distinguish these changes from the acute necrotizing arteritis found in the early phase of rejection.
All valves showed extensive vascularisation and thickening. There was a scanty infiltration of mononuclear cells and deposition of collagen.
The valves contained areas of very loose structures composed of fibrocytes.

Group two (dog Bd 9118, survival time 10 days).

The myocardium showed an extensive mononuclear cell infiltration. These cells, lymphocytes and blastlike lymphoid cells were mainly found around arteries and myocardial fibres. There was widespread congestion by intraluminal cell accumulation and focal rupture of the wall of small interstitial vessels was regularly seen. These ruptures explained the local hemorrhages into the surrounding tissue.
There was extensive myocytolysis as characterised by multiple foci of shrinkage, fragmentation and disappearance of myocardial fibres in these areas infiltrated by mononuclear cells.
The walls of small arteries and large arterioles demonstrated the features of necrotizing arteritis. There was infiltration with mononuclear cells and granulocytes, medial necrosis and swelling of the endothelium.
All the valves were edematous and infiltrated by mononuclear cells.

The macroscopical findings in the short term surviving dog suggest that heart failure primarily resulted from failure of the cardiac allograft as a pump. In the long term surviving dog, because the enormous amount of free fluid in the abdomen and the valvular lesions, it is likely that, apart from failure of the graft as a pump, the valvular lesions contributed to a great extent to the untimely death of the animal.

The grading of the histopathological findings of the sixteen grafts included in this study is summarized in table 3 and 4. To minimize the risk of bias, a "blind" assessment technique of grading the microscopical features was used. This analysis was performed independently by a consultant histopathologist.
The microscopy slides were handed at random to this investigator, who had no prior knowlegde about the clinical course of each animal.
The microscopical findings clearly demonstrate the virtual absence of a parenchymal type of rejection and instead the presence of major pathological lesions in the coronary arteries and the cardiac valves. All six dogs in

group one, surviving 44 days or longer, showed these features to a great extent, while similar lesions were absent in the two shorter surviving animals in this group. The major lesions in these last two grafts presented the features of parenchymal rejection as it is known in unmodified acute rejection, i.e. evidence of mononuclear cell infiltration, rupture of small interstitial vessels, myocytolysis and necrotizing arteritis.
These lesions have been described in great detail by Jongsma (63).
In the control group of eight DLA-nonidentical beagle littermates, only the two long term surviving animals of 44 and 48 days respectively, showed the changes found to be typical for chronic rejection and indicated as chronic obliterative arteritis.

The Fig. 6, 7 and 8 illustrate the typical histopathological features of acute cardiac allograft rejection in dog BD 9118, surviving 10 days after surgery.
The characteristic histopathological features of chronic cardiac allograft rejection are depicted in Fig. 9, 10, 11 and 12 as observed in dog BD 9172 of group one; survival time 132 days.

Table 3
Histopathology of cardiac allografts. Group one.

DLA typing		Identical							
Dog number		B945	B958	BD9173	B983	BD9219	BD9287	Bd9129	B9148
Survival time (days)		247	164	132	80	47	44	38	21
MYOCARDIUM:	mononuclear infiltrate	0	1	1	1	1	1	2	1
	myocytolysis	0	0	0	1	1	0	2	1
	necrotizing arteritis	0	0	0	0	2	0	3	2
	focal necrosis	3	2	3	1	3	3	2	2
	rupture interstitial vessels	0	0	0	1	1	0	2	0
CORONARY ARTERIES:									
(medium size)	necrotizing arteritis	0	0	0	0	2	0	3	2
	obliterative arteritis	4	3	4	2	3	3	0	0
VALVES:	edema	0	0	0	0	0	0	1	1
	mononuclear infiltrate	1	1	2	2	2	1	2	2
	capillary ingrowth	1	2	2	2	2	2	0	0
	accumulation fibrous tissue	2	3	2	2	1	1	1	0

0 refers to absence of lesion; 1 to mildest and 4 to most severe form of lesion.

Table 4
Histopathology of cardiac allografts. Group two.

DLA typing		Non-identical							
Dog number		B843	BD9118	BD9149	BD982	BD997	BD961	BD9202	B200
Survival time (days)		9	10	11	11	14	17	44	48
MYOCARDIUM:	mononuclear infiltrate	2	2	3	1	3	2	1	1
	myocytolysis	2	2	3	1	3	2	1	1
	necrotizing arteritis	3	4	4	2	3	3	2	2
	focal necrosis	3	3	3	2	3	3	2	1
	rupture interstitial vessels	2	3	3	2	3	3	1	1
CORONARY ARTERIES:									
(medium size)	necrotizing arteritis	3	3	4	2	3	3	2	1
	obliterative arteritis	0	0	0	0	0	0	3	2
VALVES:	edema	1	2	2	2	2	2	1	1
	mononuclear infiltrate	1	2	2	2	1	2	3	2
	capillary ingrowth	0	0	0	0	0	0	1	1
	accumulation fibrous tissue	0	0	0	0	0	0	1	0

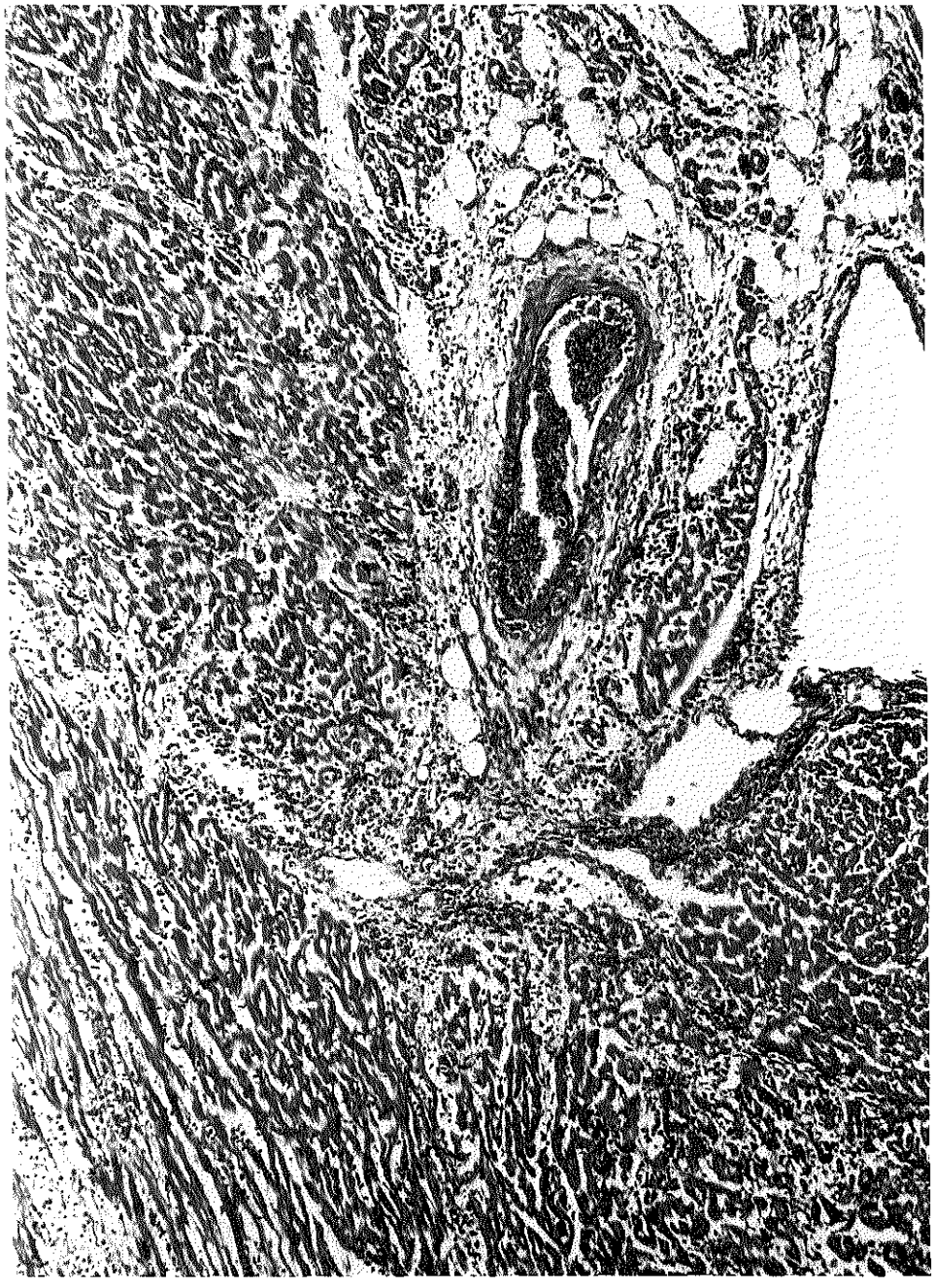


Fig. 6: Right ventricular wall, showing features of acute rejection (see text); dog BD9118, survival time 10 days. HAS.X 200.

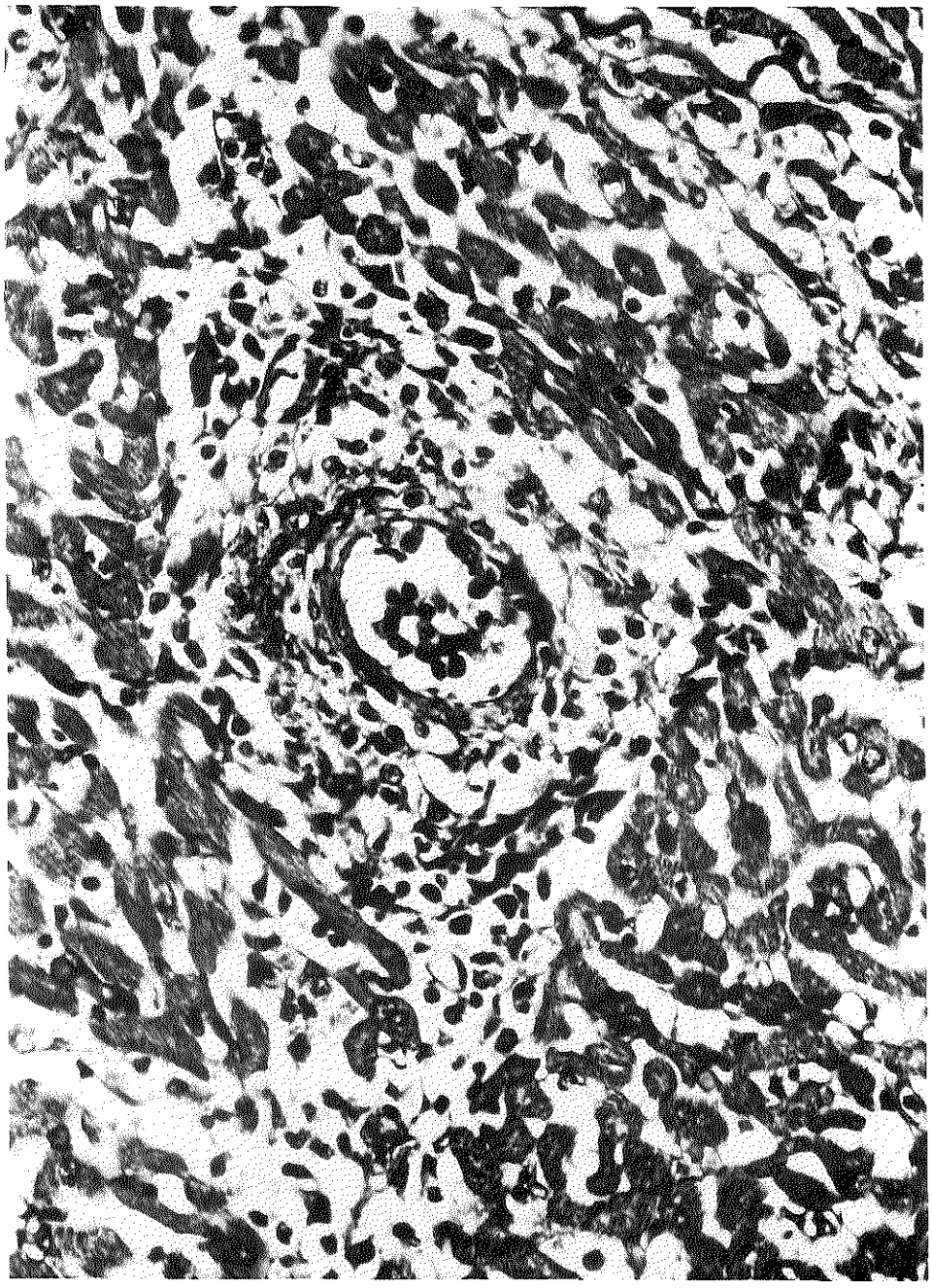


Fig. 7: Necrotizing arteritis in right vertricular wall, dog BD9118, survival time 10 days. HAS.X 510.

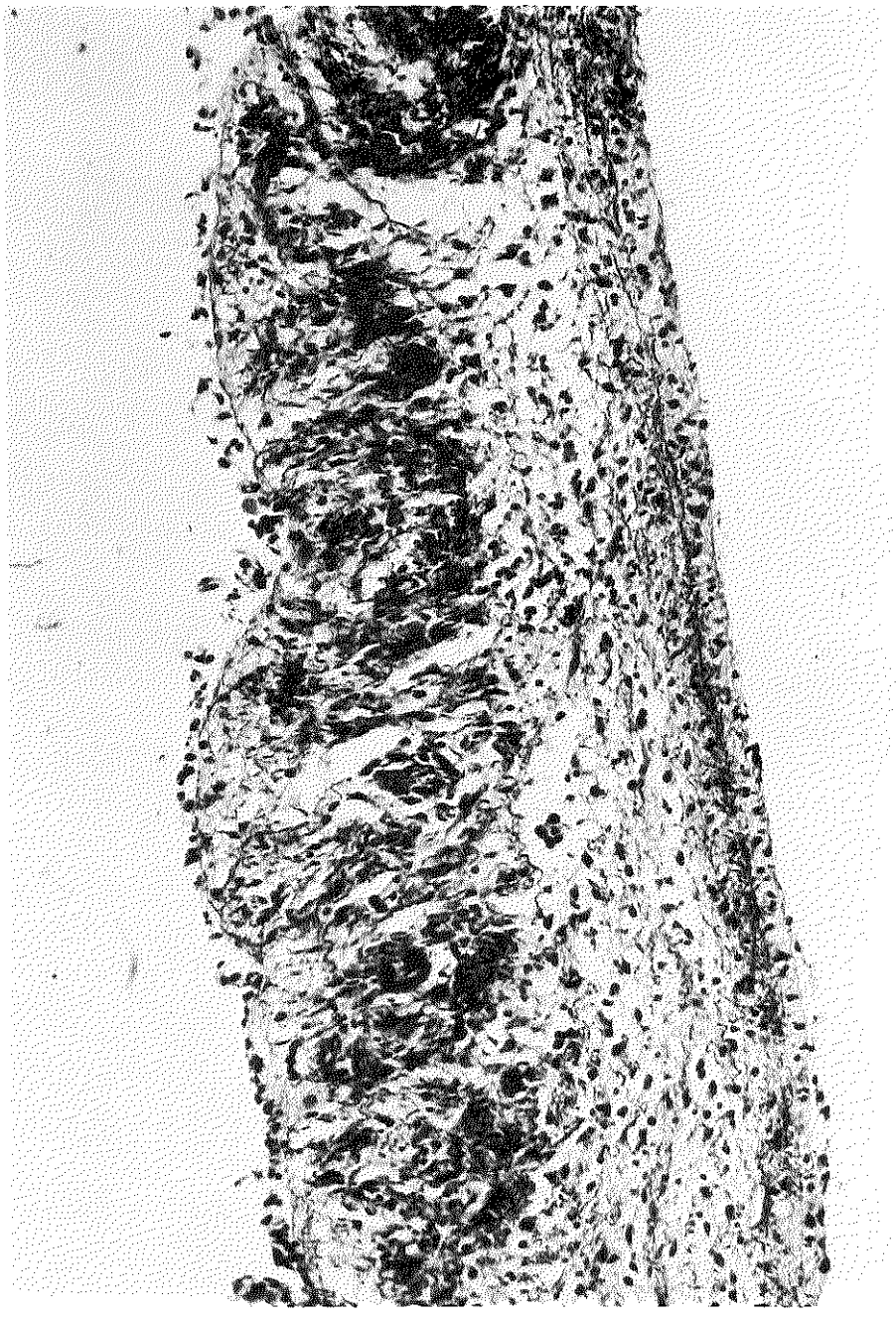


Fig. 8: Portion of mitral valve showing mononuclear infiltrate and edema, dog BD9118, survival time 10 days. HAS.X 80.

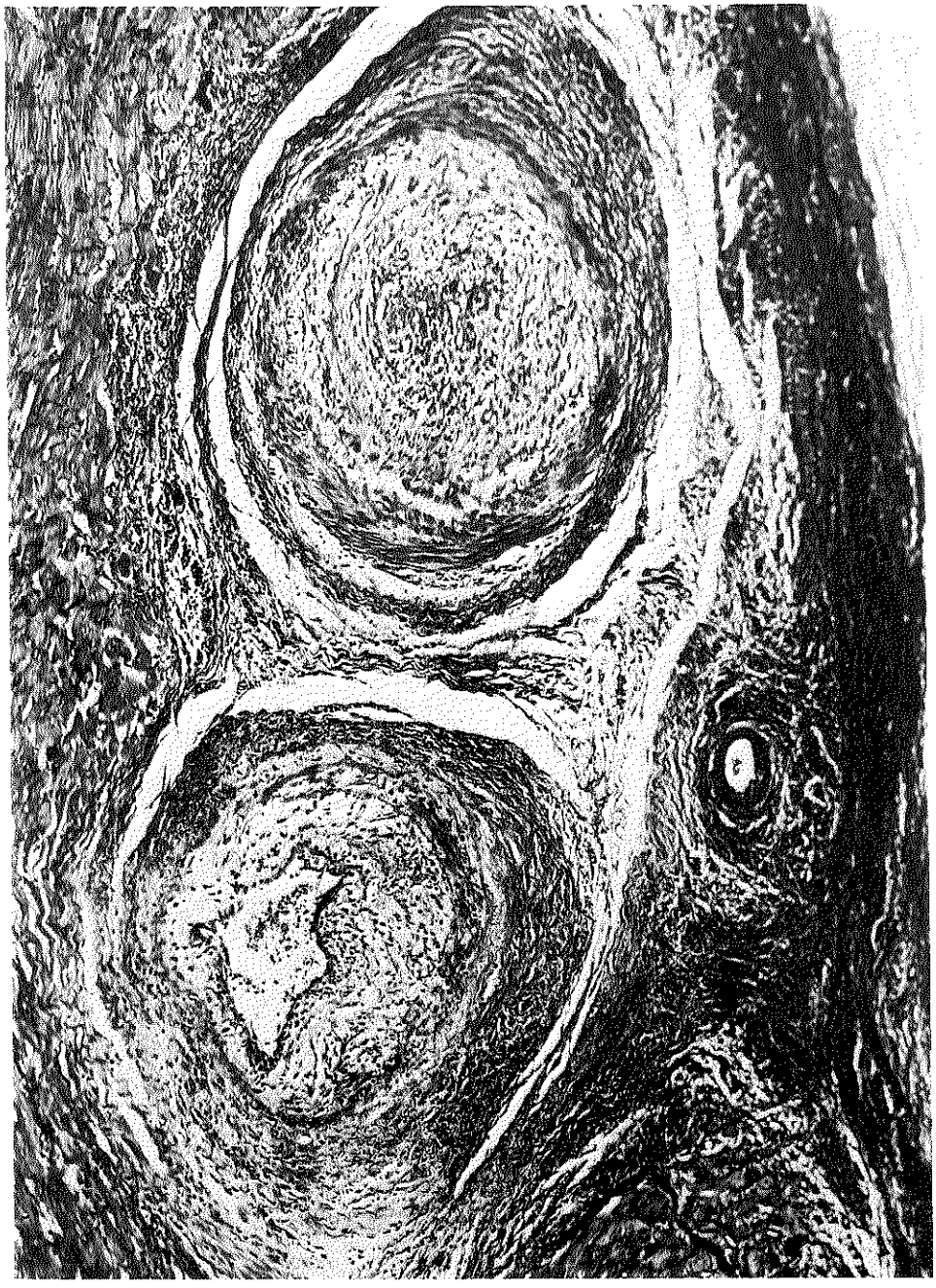


Fig. 9: Medium sized coronary arteries, dog BD9173, survival time 132 days, showing obliterative arteritis. HAS.X 80.

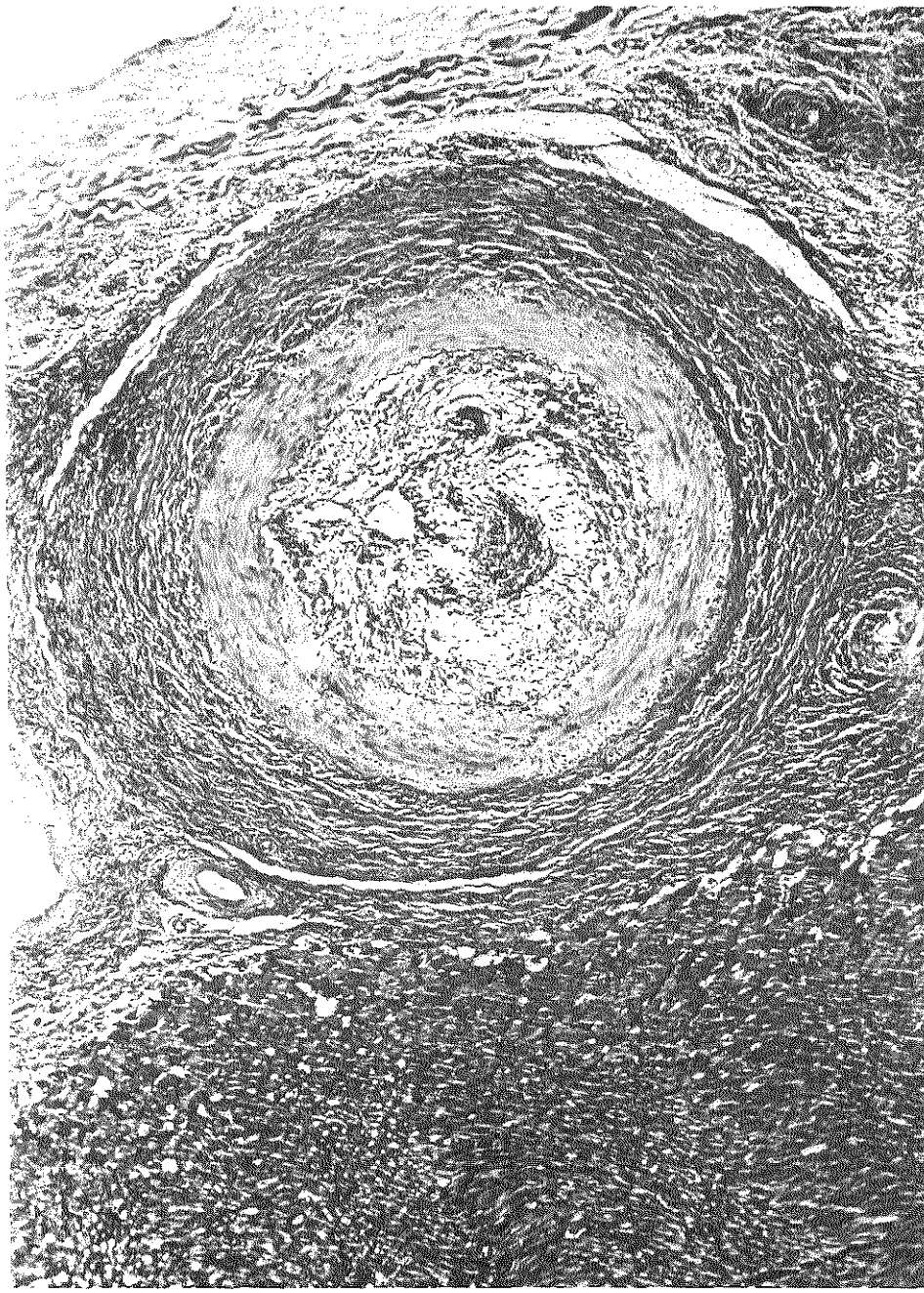


Fig. 10: Detail of medium sized coronary artery; dog BD9173, survival time 132 days: obliterative arteritis. HAS.X 200.

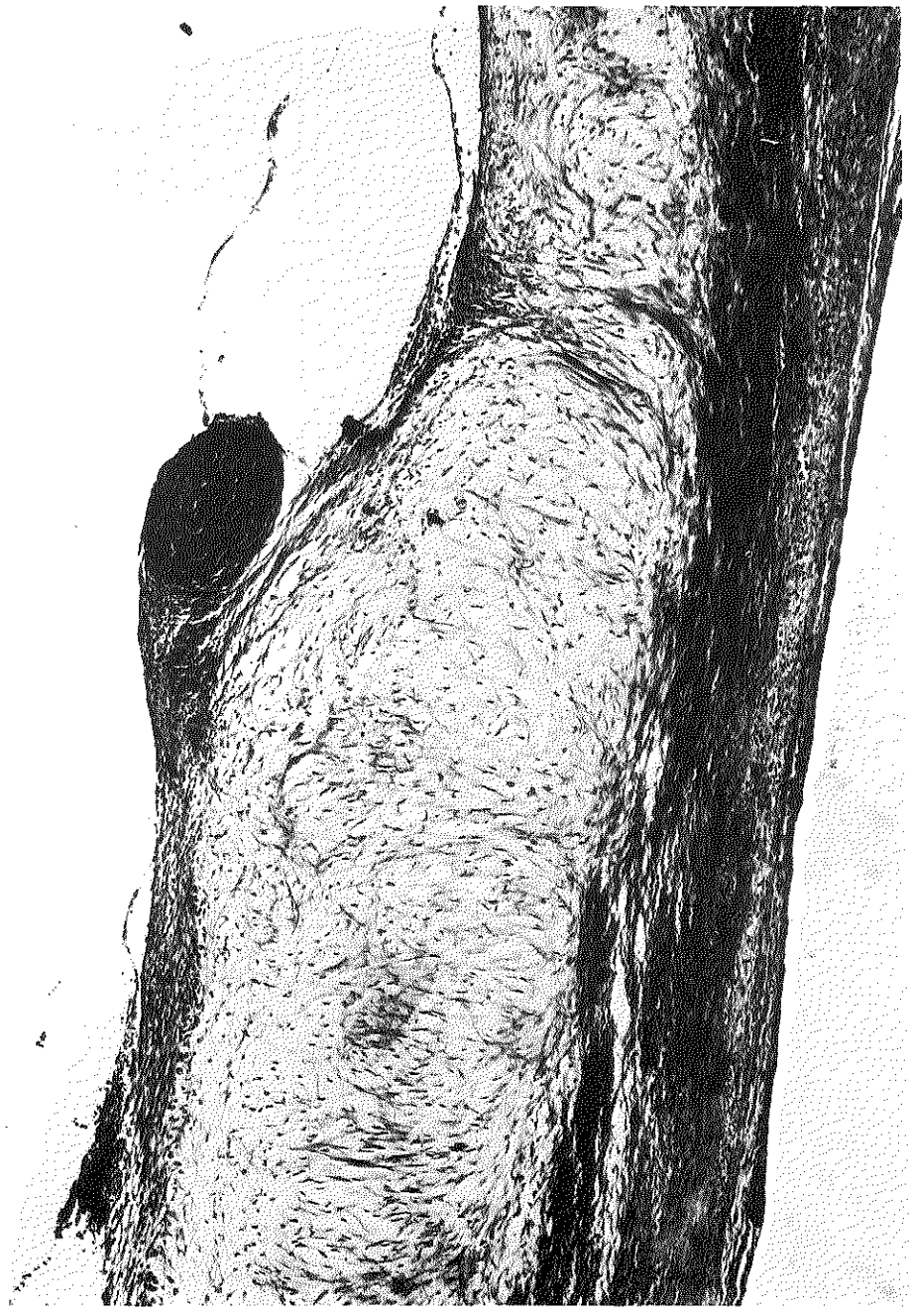


Fig. 11: Mitral valve of dog BD9173, graft survival time 132 days, showing mononuclear infiltrate, accumulation of fibrous tissue and collagen. HAS.X 80.

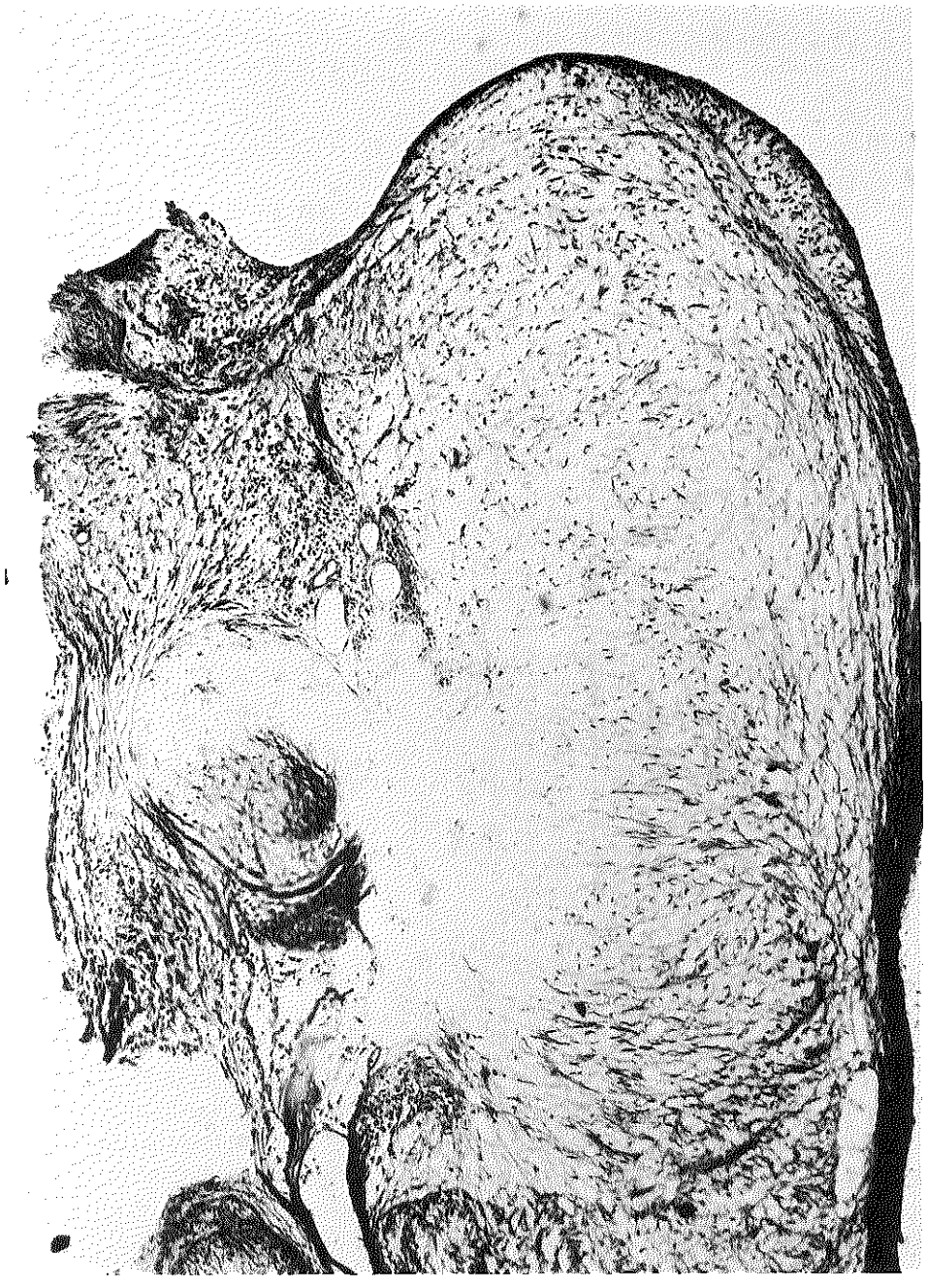


Fig. 12: Detail of mitral valve of dog BD 9173, graft survival time 132 days, showing capillary ingrowth into valve. HAS.X 80.

3.2. Second experiment.

A total number of 13 orthotopic cardiac allotransplantations, without the use of homologous third party donor blood, were performed between randomly selected unrelated mongrel dogs, matched only for size and weight. The average bodyweight of the recipients was 11.5 kg.
Excessive and uncontrollable blood loss from the aortic suture line caused the early loss of 3 dogs (23%).
The 10 surviving dogs returned to their normal activities until the process of acute allograft rejection became clinically apparent. At that time the animals became lethargic and lost their appetite.

The dog sequence number, donor and recipient sex, survival times and post-operative blood losses are listed in table 5.
Postoperative blood loss averaged 797 ml among the 10 animals and ranged from 280 ml to 2604 ml. Disregarding the excessive amount blood loss of 1300 ml and 2604 ml in two cases, the postoperative blood loss averaged 500 ml among the other eight cases.
For the ten surviving animals the mean survival time of 7.8 days (range of 6 to 9 days) was observed.

Table 5: Dog numbers, donor and recipient sex, survival times and postoperative blood loss.

	DOG NR. AND SEX	SEX OF DONOR	SURVIVAL TIME (DAYS)	POSTOPERATIVE BLOOD LOSS (cc.)
1	RH 163 M	F	9	630
2	RH 129 M	M	6	1300
3	RH 176 M	F	8	600
4	RH 161 F	F	8	420
5	RH 164 M	F	8	840
6	RH 152 M	M	7	280
7	RH 147 F	M	7	380
8	RH 136 F	M	9	2604
9	RH 153 F	M	7	455
10	RH 206 M	F	9	460

On daily ECG recordings a progressive decrease in QRS voltage, best seen in the R wave of limb lead II, was consistently found in all these dogs and preceded in every case the clinical signs of acute rejection.
At autopsy, the histopathological findings for all 10 grafts of the animals which recovered from surgery and subsequently died revealed the classical features of acute allograft rejection.

Fig. 13, depicts the hemoglobin concentration of the recipients before and after transplantation. The mean hemoglobin concentration was 9,6 mmol/l. before starting the transplantation prodedure. The combined effect of transplant surgery with the use of the heart-lung machine, hemodilution and the technique for autotransfusion caused a decrease in the hemoglobin concentration to a mean value of 6,4 mmol/l.
A minimum in hemoglobin concentration of 5.8 mmol/l was found in the early postoperative period. Afterwards it gradually increased.

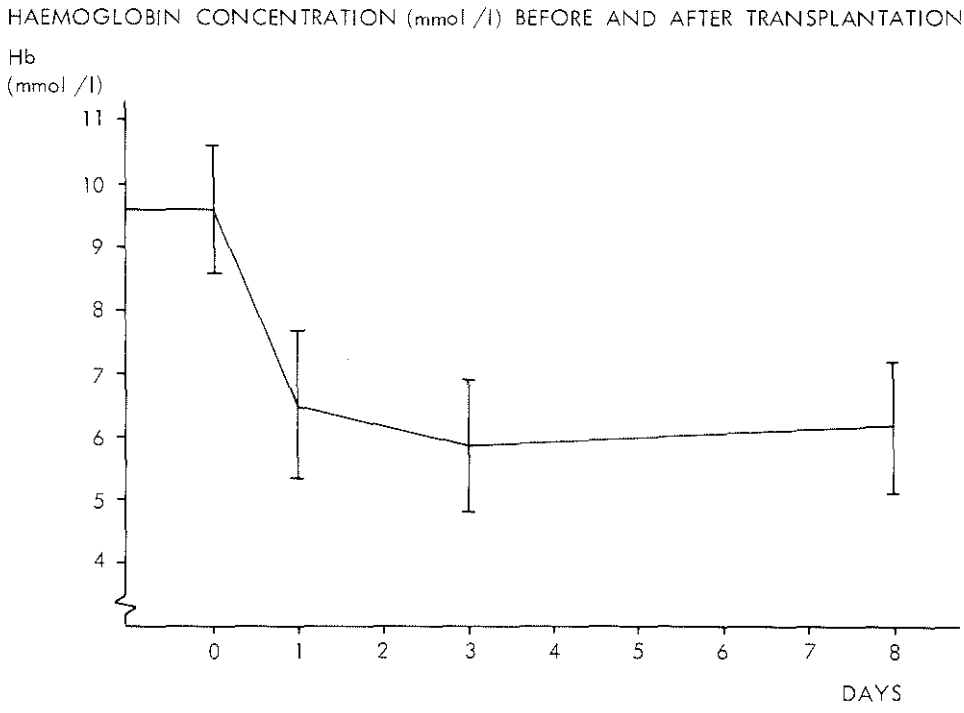


Fig. 13: Hemoglobin concentration of the recipient dogs before and after transplantation, hemodilution and autotransfusion. Mean ± standard error of the mean are given.

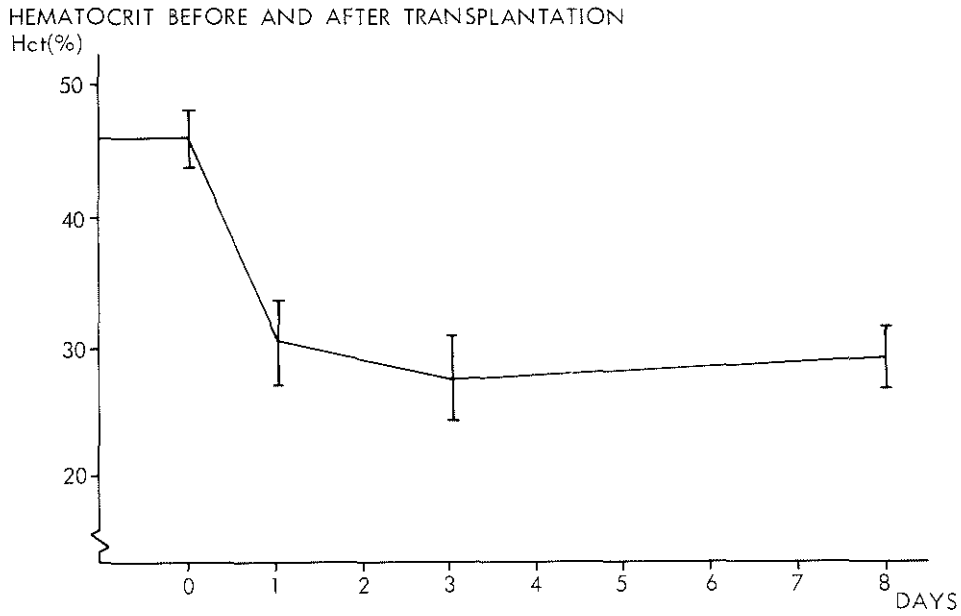


Fig. 14: Hematocrit of the recipient dogs before and after transplantation, hemodilution and autotransfusion. Mean ± standard error of the mean are given.

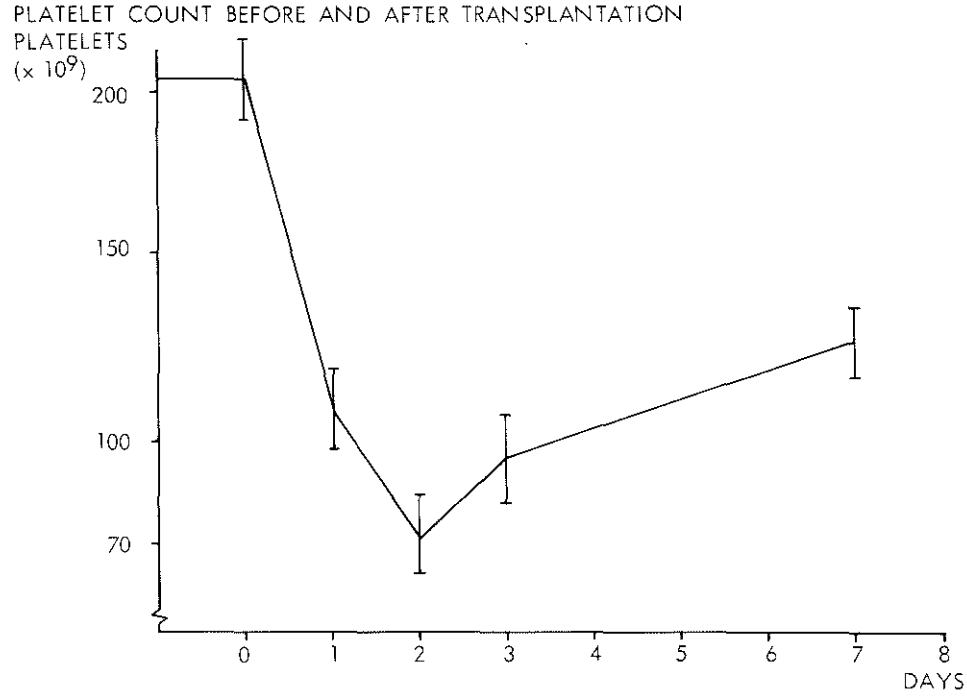


Fig. 15: Platelet count of the recipient dogs before and after transplantation, hemodilution and autotransfusion. Mean ± standard error of the mean are given.

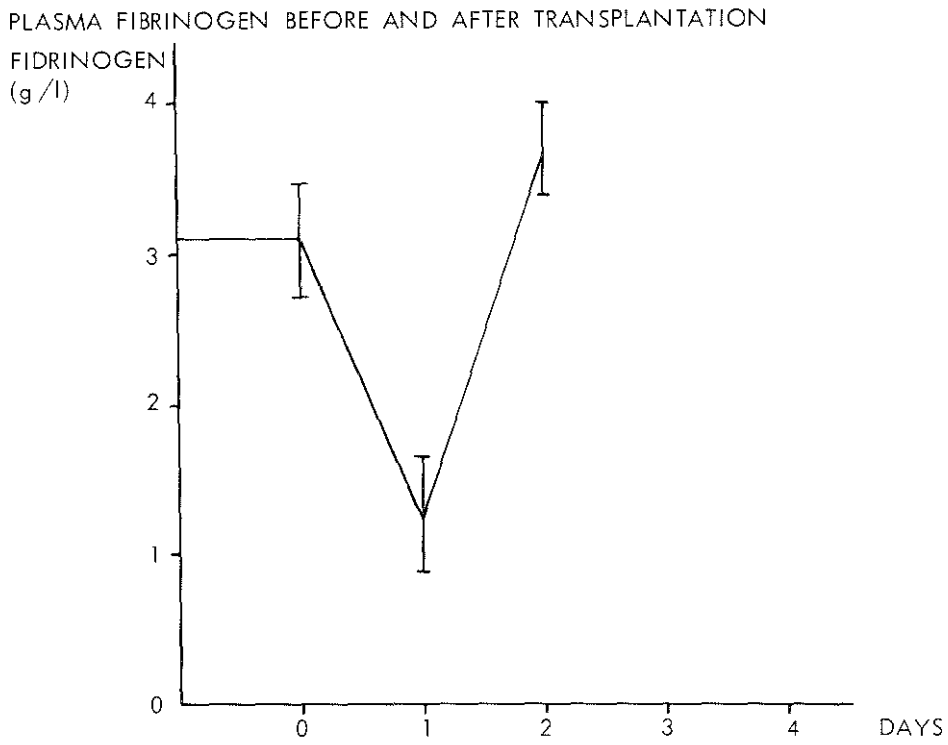


Fig. 16: Plasma fibrinogen concentration of the recipient dogs before and after transplantation, hemodilution and autotransfusion. Mean ± standard error of the mean are given.

The same pattern for the mean hematocrit of the recipients is shown in Fig. 14. Starting at a mean hematocrit of 46.7%, surgery and hemodilution caused a drop of the hematocrit to a minimum of 29.1% and then started increasing gradually again.

The platelet count before and after transplantation is demonstrated in Fig. 15 as measured by the Coulter counter technique.

Before transplantation a mean platelet count of 206.5 x 10^9/L is found. A drop of 40% is the result of the transplantation procedure and technique of autotransfusion of a mean value of 123. x 10^9/L. Also a further decrease is noticed during the first postoperative day after which day the platelet count increases again.

Plasma fibrinogen concentrations are shown in Fig. 16. Before transplantation a mean plasma fibrinogen concentration of 3.1 g/l is found in the recipients. Immediately after surgery there is a drop to a mean value of 1.3 g/l. However, during the first postoperative day the mean fibrinogen concentration increased to above pretransplant levels at 4.1 g/l. Fibrinogen degeneration products could never be demonstrated.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.1. First experiment.

The first objective of this study as formulated in chapter 1.3, has been accomplished. According to the results documented in the preceding chapter a prolongation of canine cardiac allograft survival can be repeatedly produced without the use of immunosuppressive treatment.

DLA matching in sibs and prolongation of cardiac allograft survival.

In the first chapter it is argued that both a prolongation of allograft survival and the absence of an immunosuppressive treatment are judged to be essential prerequisites in producing a canine model of chronic rejection of a cardiac allograft. The term "chronic" merely indicates the importance of a lapse of time to permit a slow development of the rejection process after allografting. This is in contrast to the process of acute allograft rejection which, without appropriate treatment, will result in a short duration of allograft survival or, in the case of late acute rejection, when death of the animal ensues in a few days.
The process of destruction of an allograft, i.e. the whole chain of events aimed at antigen localization, destruction and removal is only partly known (125); it is caused by the immune reaction of the recipient against the antigenic challenge of an allograft. The intensity of the unmodified immune reaction is determined by the antigeneticity of the graft and the intensity of the cellular and humoral immune response of the host. As a consequence, the differences in strong and weak transplantation or histocompatibility antigens between donor organ and recipient influence the intensity of the immune reaction.
Minimizing the disparity of these histocompatibility antigens between donor

organ and host tissue explains why DLA identity has a profound influence on graft survival if donor and recipient are siblings. Also a good match is accomplished for the other undefined gene products of the major histocompatibility complex (102).
As for the cardiac allograft (14, 96, 99), DLA matching in siblings has also been demonstrated by many transplantation experiments to influence graft survival, such as skin, kidney, small intestine, pancreas, lung, liver, bone marrow and frozen nerve (1, 11, 12, 32, 98, 110, 111, 112, 122, 123). The intensity of the immune reaction is also determined by presently undefined histocompatibility systems, other than the DLA histocompatibility complex (19, 120, 121). In other words, the present level of understanding the DLA-serology, does not necessarily signify the complete absence of incompatibility between donor and recipient. Therefore, DLA-identity between donor and recipient siblings can not, by the presence of these minor histo-incompatibilities, prevent the ultimate rejection of a graft by its host.
It has been demonstrated in mice that, where weaker histo-incompatibilities are involved between donor organ and host tissue, a greater variability in survival times is to be expected (104). In fact, this very likely is an important reason for the observation in group one of this study, where graft survival time ranges from 21 to 247 days (mean survival time: 96.6 days). Accordingly, this could also explain the observation in the second group of DLA-nonidentical beagle littermates after cardiac allotransplantation, that two long term survivors (respectively 48 and 44 days) and 6 short term survivors (mean survival time: 12 days; range 9 to 17 days) were documented.

A comparison of the survival times of different visceral organs after transplantation is a difficult problem. Many factors are involved which could make a comparison invalid. These include differences in the antigenicity of the organs, the type of transplant surgery involved, the differences in the physiologic tasks of these organs and so on. Without taking these factors into account, it is remarkable that for instance the survival times for cardiac allografts documented in group one of this study, exceed those of kidney allografts between DLA-identical sibs (mean survival time 43.0 days). These data have been reported by Westbroek et al. from the same laboratory (123). This disparity in survival times between the two transplanted organs could be explained by a beneficial effect of the liberal use of third party donor blood in cardiac allografting during the operative period. Homologous

donor blood was not administered to the recipients of the kidney transplants. This interesting aspect of allografting stresses again the urgent need to develop a suitable model for orthotopic heart transplantation in dogs without the use of homologous blood.

Donor-recipient incompatibilities for Swisher erythrocyte antigens (113) were not taken into account in our study. However, the results of Rappaport et al. (99) did not show direct evidence that such incompatibilities had an adverse influence upon the duration of cardiac allograft survival in tissue typed beagle littermates.

A detailed discussion on the many factors that influence the immune response of a recipient against its allograft is beyond the scope of this study. However, the occasional finding of a prolongation of survival time in puppies after cardiac allotransplantation without the use of immunosuppressive drug should be mentioned.
A survival time of 150 days after orthotopic heart transplantation between littermate puppies has been reported by Hurley and Kosek (61), but they stated that no tissue typing of donor and recipient was performed. DLA-identity of donor and recipient could have explained this result.
Kondo et al. (65, 66) documented two long term survivors after orthotopic heart transplantation in puppies. Donor and recipient were unrelated. Neither dog received immunosuppressive treatment. One animal was well and alive 112 days after surgery at the time of report and the other dog survived 57 days.
Although the authors state that these results may be explained by "unpredictable" histocompatibility, they also express the belief that young animals tolerate allografts better than adults.
For kidney transplants Claman et al. (33) reported one long term survivor of 68 days in 11 experiments after allografting from a mongrel puppy to an unrelated adult mongrel dog. They suggest that the concept of using "immature" tissue needs further exploration.

The electrocardiogram and cardiac allograft survival.

The arrhythmias, present during the early postoperative phase are very likely the result of surgical trauma, the use of topical hypothermia for pre-

servation of the heart during the surgical procedure and the period of anoxic arrest in itself.
The arrhythmias receded spontaneously without producing obvious alterations in the clinical status of the animals. These transient arrhythmias are unlikely to be related to any type of the rejection response by the host against the cardiac allograft.

Many experimental and clinical studies have shown a relationship between the late occurence of electrocardiographic abnormalities and the pathological changes in the transplanted heart during the course of the rejection process (47, 80, 109). Above all, a decrease in the QRS voltage proved to be the most consistent and an early sign of the acute rejection process, usually beginning four to five days prior to the death of the recipient (80). Unless immunosuppressive drugs are given, the decrease in the QRS voltage is progressive during the final course of acute rejection (47, 79). In clinical practice this close correlation between a decrease in the QRS voltage and the presence of acute rejection could be verified by the method of taking endomyocardial biopsies (27, 49).
The cardiac conduction system is as vulnerable to immunologic damage as the other tissues of the heart, but at present, the most likely primary factor to explain this phenomenon of a drop in the QRS voltage is the progression of myocardial edema during the acute rejection process (10).
The follow-up studies of the short term surviving dogs in group one and group two confirm these observations:

1. the QRS voltage drop was the most consistent finding in the short surviving dogs in both groups.
2. it was not observed in the long term surviving animals in both groups.
3. it correlated well with the histopathological findings of acute cardiac allograft rejection.
4. in every instance there was a short time interval between the first recording of this electrocardiographic abnormality and the time of death of the animal.

The initial electrocardiographic changes observed in the long term surviving dogs were arrhythmias, mainly consisting of conduction disturbances. In all instances these were followed by a prolonged time interval between the initial recording of the electrocardiographic abnormality and the time of death of the animal as compared to the short time interval in the case of acute rejection.

The problem of explaining these arrhythmias and the prolonged time interval between their first recording and the time of death of the animal was not explored in this study. Certainly, it can be postulated that progressive ischemic damage to the myocardium and the cardiac conduction system, as the result of the vascular lesions observed in the graft, is a major cause. At least it signifies that irreversible and localized damage to the myocardium has occurred. The method of taking serial myocardial biopsies (95), which was not used in this study, could be expected to contribute to the detection of the "point of no return" of these lesions.

Histopathology and cardiac allograft survival.

The predominant histopathological feature of chronic cardiac allograft rejection is to be found in the larger medium sized vessels of the graft and is indicated as *chronic obliterative arteritis.* This was repeatedly found in the grafts of the long term surviving animals of group one and in the two long surviving animals of group two. This observation could be convincingly confirmed by the "blind" assessment technique in grading the lesions by an independent investigator.
He also confirmed the observation that the obliterative vascular lesions were never observed in the grafts of the short term surviving animals.
Three dogs, Bd 9219 in group one and Bd 9202, B 200 in group two (tables 3 and 4, page 38, 39) demonstrated both types of vascular lesions, i.e. necrotizing and obliterative arteritis.
Observing the extremes and the inbetweens of the dogs in group one and group two brought together in one serie, then there is obviously a complete spectrum of lesions. Lesions of the parenchymal type are most promiment in the shortest surviving animals, an overlap of parenchymal and vascular lesions occurs in the middle and the obliterative vascular lesions are the most prominent features in the long term surviving animals.

Concentrating on *chronic obliterative arteritis* as the prominent lesion in this model of chronic rejection of the orthotopic canine cardiac allograft, the possible explanations for the mechanism causing this lesion should be discussed.
A spectrum of lesions, as observed in the whole serie of dogs in group one plus group two, favors the concept of quantitative differences in the immune response of the hosts. The severity of the vascular lesions parallels the

duration of graft survival and therefore also favors the concept of quantitative differences.
As prolongation of graft survival resulted from DLA matching, it is therefore unlikely to be the result of qualitative changes in the host immune response.
Adequate immunosuppressive treatment of acute rejection episodes in a long term surviving dog produces the same type of lesions typical of chronic rejection in the graft as has been reported by many investigators (68, 69, 70, 75).
These arguments all support the concept that chronic rejection of a cardiac allograft from a DLA-identical donor in this model is most likely explained by quantitative rather than by qualitative changes in the humoral and cellular immune response of the recipient.
It is postulated that the humoral or antibody-mediated immune response is mainly responsible for these vascular lesions (73, 92, 117, 124).
The intensity of this part of the immune response does not necessarily have to be of the low grade type, but its typical features can be accentuated by a low grade course of the cell-mediated immunologic injury over a long period of time.
The precise mechanism of the humoral immune response causing the vascular lesions is still much debated. Even a certain synergism between the humoral and cellular components can not be excluded (104).
Based on an analysis of the cell specificity of the antibody response in human kidney allograft rejection, Cerelli et al. (29, 30) postulated that histocompatibility antigens are found on the vascular endothelial cells, but they also contain antigen not found on renal cells or lymphocytes. It is their opinion that this antigen is important in the pathogenesis of the vascular lesions in a graft.
It has also been shown that during the rejection process there is a deposition of antigen-antibody complexes in the vascular wall (59, 62, 82, 97). These immuno-complexes are supposed to damage the intima of the small vessel wall by provoking platelet aggregation.
O' Connell and Mowbray (91) succeeded in provoking such arterial intima lesions in rabbits.
Kosek et al. (70) have suggested a unifying hypothesis to explain the similarity between chronic obliterative arteritis and spontaneous arteriosclerosis. They state that the primary immune damage to the vasa vasorum of the arteries leads to medial hypoxia. Also there is an immune damage direct to the endothelial cells of the arteries. The whole process results in a sub-

endothelial accumulation of myointimal cells which migrate inward to restore the damaged endothelial surface of the arteries.

The *Valvular lesions*, as they are described in this study and observed in the long term surviving animals were also reported by Hurley et al. (61) in one dog surviving 5 months without immunosuppressive treatment.
Kosek et al. (68) reported segmental replacement by hyaline scleroprotein in the valves in four out of 12 dogs surviving 2 months or longer with immunosuppressive treatment.
Leandri-Césari et al (75) described thickening and irregularities of the free edges of the valves in a dog surviving seven years after orthotopic heart transplantation. However, the authors did not report on the microscopic features of the valves.
Bieber et al. (10), in their article on cardiac allograft pathology in man, mention a mild degree of mitral and tricuspid subvalvular fibrosis in the two oldest specimens.
It can be postulated that the valvular lesions observed in this study are basically the result of the humoral or antibody-mediated immune response of the host as it is also responsible for the vascular lesions in the graft.

In general, it is obvious that the histopathological changes of chronic rejection represent essentially an irreversible state of rejection. Because no immunosuppressive drugs were employed and no late acute rejection was superimposed in this model of chronic rejection, the orthotopic canine cardiac allograft from a DLA-identical donor can be used as a standardized model of chronic rejection. At least three applications are apparent.

1. To study the course and the characteristic features of chronic rejection in more detail. Our study did not document the sequential histologic events as they occur in the cardiac graft.
   No helpfull parameters for monitoring the early onset of the observed changes emerged from our study.
   Studies have been undertaken in our laboratory and reported by Penn et al. (96) to enlighten these aspects of chronic rejection in this canine model.
2. To study the aspecific suppression of the immune response by the administration of drugs during the course of chronic rejection in a standardized model, not superimposed by early or late acute rejection. The same holds for the study of specific suppression of the immune response, for example, studies on enhancement of the cardiac allograft in this model.

3. To study the possible influence (48, 73) on the progress of the characteristic arterial intimal changes during chronic rejection or to prevent their occurrence by treating the recipient with anticoagulants or a well balanced diet.
   Accepting Kosek's unifying hypothesis, such a study could contribute to a large extent to our knowledge of spontaneous arteriosclerosis.

The most important *shortcoming of this study* has been the use of homologous third party donor blood during the surgical procedure and postoperative course in this first experiment. The relevance of blood transfusions has already been mentioned and will be included in the discussion on the results of the second experiment.

4.2. Second experiment.

The results presented in chapter 3.2 clearly demonstrate the technical feasibility of performing orthotopic canine cardiac allotransplantation without administering third party donor blood to the recipient. In spite of the low average weight of the recipients (11.5 kg), the methods of total hemodilution and autotransfusion proved to be adequate to achieve a survival rate of 77%.
The *choice of small sized mongrel dogs* in this study was made to enable the planning of new transplantation experiments in relatively small sized but more costly beagles. This is in accordance with the general tendency to standardize the choice of the experimental dog in transplantation experiments to make the comparison of the results more meaningfull. To this end the small sized beagle is more and more the animal of choice and is used nowadays in many laboratories (1, 2, 85, 115).
The *surgical mortality* of 23% in this experiment was even less than the 29% observed in the first experiment. However, it is premature to conclude from these data that total hemodilution and postoperative autotransfusion is superior to the use of third party donor blood.
There has been a notable time gap between the performance of the surgical procedures of the two experiments. Undoubtedly a gain in experience contributed a great deal to the disparity in surgical mortality between the two experiments. Nevertheless, the friable supravalvular aortic tissue again was the most challenging problem of the surgery.

There is still much debate about the optimal amount of *hemodilution* in clinical practice. The opinions diverge from a complete blood priming of the heart lung machine to non-blood priming or so called total hemodilution (36, 41, 126).
The low hemoglobin levels resulting from total hemodilution could endanger the myocardium by producing ischemia. Brazier et al. (16) demonstrated that in anesthetized dogs with normal coronary arteries, coronary bloodflow remains normally distributed and myocardial oxygen delivery is adequately maintained with moderately severe levels of normovolemic anemia, i.e. hemoglobin concentration above 3.0 mmol per liter.
Such a low level of hemoglobin concentration was never reached in our experiments.
Mullerworth et al. (86) have reported a study on the use of HAEMACCEL®, a degraded and repolymerized bovine gelatin, for total pump prime during 60 minutes of cardiopulmonary bypass in five dogs weighing 18 to 23 kg. No major surgery was performed and no blood transfusions were administered. All animals survived the procedure, appeared healthy and no lasting ill effects were detected. By measuring clotting time, partial thromboplastin time, prothrombin time, and euglobulinolysis they observed a slight decrease in clotting efficiency which was demonstrated most clearly by the partial thromboplastin time. Gross clotting disturbances were also not observed in our experiments, even though major surgery was added to the procedure.

The idea of *autotransfusion* first came into history for a quite different reason than it was used in this experiment. The first autotransfusion can be traced back to the beginning of the last century (3).
To salvage the loss of shed blood and not having the opportunity this loss to be replaced by donating homologous blood transfusions, the obvious solution was autotransfusion. With the development of blood banks it became an obsolete procedure until the last decade when interest has increased again for the use of this technique in the treatment of patients with massive bleeding. Numerous articles, mainly on intraoperative autotransfusion, have since then appeared. At least nine different autotransfusion devices have been evaluated experimentally and clinically (84). The first autotransfusion seminar was organized by Brawly and Hauer at the John Hopkins University School of Medicin, Baltimore, in April 1978 (15).
Intracardiac surgery, routinely employing cardiopulmonary bypass, is completely dependent on the principles of intraoperative autotransfusion.

The complexity of the hematological problems involved are tremendous and many aspects of it are still unexplained.
The most recently devised technique of autotransfusion for the utilization of spilled blood in closed cavities during the postoperative period is less commonly used however.
Symbass recently (114) reported on the effect of autotransfusion upon the blood components and the recipient. In dogs he connected a pleural space to a femoral artery with a plastic tubing and a chest tube was inserted into the pleural space. With the chest tube clamped, one quarter of the dog's blood volume was allowed to bleed into the right pleural space. No ACD-drip by an infusion line into the pleural space was used. After autotransfusion from the collecting bottle, he found no changes in the dog's bleeding and clotting times. The hematocrits of the blood drawn from the hemothorax were significantly lower than that of the control arterial blood. Platelets and plasma fibrinogen decreased or almost vanished from the hemothorax. However, after autotransfusion, both parameters were at or above the control arterial levels. He postulated that the trapping of red cells by intrathoracic clot formation and the defibrinating mechanical action of the lung and heart were the major causes of the decrease in hematocrits, platelets and plasma fibrinogen. Because of these low values it is very likely that the blood from the hemothorax did not clot. On the next day the plasma fibrinogen concentration was above baseline level. He assumed that the occurrence of hemolysis as a result of the autotransfusion technique, explained these high levels of plasma fibrinogen. However, it has also been stated (9) that the body is able to compensate fully for the deficiency in plasma fibrinogen.
The important advantage of this technique of autotransfusion is that anticoagulation does not have to be employed. In our experiment we added an infusion line for a continuous ACD-drip into the thoracic cavities, as has been practiced by other investigators (18). This is supposed to prevent clot formation in the chest and allows the optimal use of the shed blood.
In general, our data on hemoglobin concentration, hematocrit, platelet count and plasma fibrinogen are in accordance with the data reported by Symbass. The below normal levels of hemoglobin concentration, hematocrit and platelet count found after surgery are well explained by the low values in the autotransfusion blood which is centrifugated and returned to the recipient as packed cells. The waste of blood during the transplant surgery, which also includes the use of the non-blood primed heart lung machine contributed to this. The elimination of the hemolytic plasma by centrifuga-

tion of the collected blood, did not prevent the occurrence of above normal values in plasma fibrinogen as measured on the second postoperative day. Fibrinogen degeneration products could never be demonstrated in the autotransfused recipients. This observation is also in accordance with data reported in literature (9).
The results demonstrate that an average postoperative blood loss of 500 ml, which is more than half of the estimated mean blood volume of 900 ml in these dogs, can be successfully handled by autotransfusion. Even the excessive amount of shed blood in two instances, 1300 and 2600 ml respectively, could be effectively managed by this simple method.

After surgery the animals showed normal activities comparable to the animals in the first experiment. However, it should be mentioned that they were limited in physical activities by their cages and were not submitted to any type of stress test.
Acute rejection of the graft was heralded by a progressive decrease in the R wave of limblead II in all cases and preceeded the clinical signs of lethargy and anorexia.
The histopathological graft findings demonstrated the typical features of acute cardiac allograft rejection. These findings did not reveal any differences compared to the histopathology of the acute rejected grafts in the series of orthotopic heart transplants performed in unrelated mongrels, with the use of third party donor blood published by Jongsma (63).

The topic of *blood transfusion and transplantation* has been recently overviewed by van Rood and Balner and by Morris (103).
The issues at stake are, on the one hand a proven detrimental effect (64) and on the other hand, a claimed beneficial effect of blood transfusions on the prognosis of graft survival (60, 94). The article of Opelz et all. (93) triggered the discussions on this topic. They reported that patients, not having had pretransplant blood transfusion, fared worse than patients that did receive blood transfusions before the kidney transplant was inserted.
At about the same time Coulson et al. (39) formulated the effect of previous blood transfusion on graft prognosis in clinical heart transplantation from the opposite point of view. They stated "It appears that patients who have had previous heart surgery on (cardio-pulmonary) bypass fare better with their heart transplant than those who have not had surgery". It is suggested by these authors that serum factors are produced in the patient and inhibit or depress lymphocyte responsiveness in vitro. The nature of these serum

factors is not clear but when added to mixed lymphocyte cultures these sera exerted an inhibitory effect. To these authors there appeared to be a correlation between transplant survival and the presence of serum immunodepressive factors.
In kidney transplantation it is discussed that blood transfusions could distinguish between those patients with an intense immune response (responders) and those with a reduced immune response (nonresponders) (17, 19).
In rodents the beneficial effect of the administration of blood on graft prognosis prior to transplantation has been reported some years ago by Marquet et al (83). Other experiments in different species like dogs and monkeys (46, 90) have been reported to favor a beneficial effect of pretransplant transfusions.
In general all investigators, interested in this aspect of organ transplantation, admit to a lack of complete knowledge about the precise mechanism that causes a favorable influence on graft prognosis on the one hand and an acceleration of graft rejection on the other hand.
According to Obertop (90) a definite favorable influence on canine kidney graft prognosis is observed when pretransplant third party bloodtransfusions are combined with the use of immunosuppressive drugs after surgery.

It is tempting to compare the observed survival times in this second experiment (mean survival time 7.8 days; S.D. ± 1.0) as presented in table 5 with the observed survival times reported by Jongsma (63). In his serie of 13 unmodified orthotopic cardiac allografts in unrelated mongrels the acute rejection response resulted in the following survival times: respectively 5, 5, 5, 6, 9, 9, 10, 10, 10, 11, 15, 15, 15 days (mean survival time 9.6 days; S.D. ± 3.7).
A comparison of the two series appears to be appropriate because all transplant procedures in both series were performed according to the same surgical technique and by the same surgical team. Assuming the validity of comparing the two series while no data on DLA matching are available, the major disparity between the two series is the use of third party donor blood. There is no statistically significant difference between the mean survival times of the two series.
In Fig. 17 the comparison of these two series is depicted graphically by the survival curves of the grafts in these two series. This figure suggests an influence of the use of homologous donor blood on the outcome of an orthotopic canine cardiac allograft, in the sense that the administration of

donor blood tends to prolong graft survival time in a number of dogs. Out of 13 dogs in the serie of Jongsma 7 animals (54%) lived 1 to 5 days longer than the longest survivor of 9 days in the serie of our second experiment.

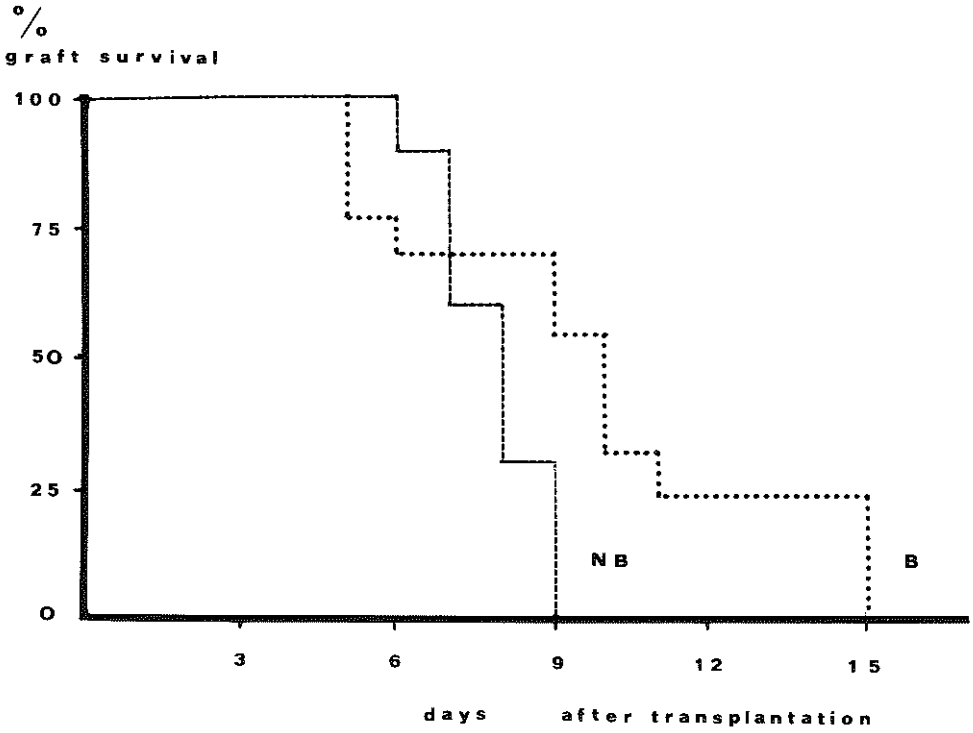


Fig. 17 Orthotopic cardiac allograft survival in 13 mongrel dogs with the use of homologous donor blood (B) and 10 mongrel dogs without the use of homologous donor blood (NB).

Certainly prospective experiments with regard to the effects of third party donor blood on graft prognosis in orthotopic cardiac transplantation in well controlled studies have to be performed. This has been the very reason to formulate and to undertake the second objective of the present study. This model of orthotopic canine cardiac allografting without the use of homologous donor blood is now available and enables the design of experiments in well controlled studies. Schemes of pretreatment of the recipient with whole blood or blood components to study the effect on the outcome of an orthotopic cardiac allograft are now within reach.

The Hatter was the first to break the silence.
"Have you guessed the riddle yet?" he asked.
"No, I give it up", Alice replied: "what is the answer?"
"I haven't the slightest idea", said the Hatter.
"Nor I", said the March Hare.

A MAD TEA-PARTY.
Alice in Wonderland.

SUMMARY

In the first part of Chapter One it is argued that progress with regard to long term results in experimental and clinical heart transplantation is limited by the late development of graft arteriosclerosis. This is the result of an insidious, slowly developing process of chronic rejection. In contrast to acute rejection, the histopathological changes occurring in the vasculature of the cardiac allograft during chronic rejection proved to be progressive and not amenable to be reversed by the use of high dosages of the presently available immunosuppressive drugs. So far, only precautionary measures like the administration of anticoagulants and the prescription of dietary restrictions to the heart transplant patient are claimed to be used with some success against the process of chronic rejection.

It is concluded that, to enable the study in more detail of the ongoing events during chronic rejection, a search for a suitable model in canine orthotopic cardiac allografting should be undertaken.
In the rationale of the study (paragraph 1.2) the arguments are presented that preferable such a model should not be influenced by the detrimental effects of immunosuppressive drugs. It also reasoned that the use of homologous third party donor blood during transplant surgery and thereby its influence on graft prognosis must be taken into account.
In paragraph 1.3 the objectives of the study are formulated as follows:
The first objective was to develop a model of chronic rejection of an orthotopic canine cardiac allograft using DLA-identical beagle littermates without immunosuppressive drugs.
The second objective was to design and test a surgical technique of orthotopic cardiac transplantation without the need for homologous donor blood.
In Chapter Two the data, relevant to the material and methods used in this study, are presented and is followed by the documentation of the results in the Third Chapter.
These results demonstrate that without the use of immunosuppressive drugs, DLA-identity between donor and recipient beagle siblings after orthotopic heart transplantation provides the characteristic histopathological feature of chronic rejection, indicated as chronic obliterative arteritis.

Paragraph 3.2 of the Third Chapter documents the technical feasibility of performing orthotopic canine cardiac allotransplantation without the use of homologous third party donor blood.
In the first part of Chapter Four the obtained canine model of chronic rejection of an orthotopic heart transplant is discussed and a number of applications for its use are mentioned.
The second part of Chapter Four comprises a more detailed discussion on the use of total hemodilution and autotransfusion for successfull orthotopic heart transplantation in a small sized dog.
Finally, after a brief discussion on the relationship between blood transfusions and transplantation, a tentative comparison is made between the survival times of the ten dogs observed in the second experiment with the survival times of thirteen dogs operated in the past by the same surgical team under comparable circumstances but with the liberal use of homologous donor blood during transplant surgery. This comparison suggests that the administration of homologous third party donor blood during transplant surgery tends to prolong graft survival time.

SAMENVATTING.

In de eerste paragraaf van Hoofdstuk 1 wordt getracht duidelijk te maken dat een verbetering van de resultaten op langere termijn bij de experimentele en klinische harttransplantatie gehinderd wordt door het late optreden van arteriosclerose in het transplantaat.
Dit is het resultaat van een zich geleidelijk ontwikkelend proces van chronische transplantaat afstoting. De histopathologische veranderingen, welke optreden in het vaatstelsel van het harttransplantaat gedurende de chronische afstoting blijken, in tegenstelling tot de afwijkingen bij de acute transplantaat afstoting, progressief te zijn ondanks het gebruik van immunosuppressiva en niet reversibel bij hogere doseringen van deze medicamenten. Alleen de toediening van anticoagulantia en het voorschrijven van dieet maatregelen zijn tot op heden met enig succes toegepast tegen het optreden van de chronische transplantaat afstoting.
Om een meer gedetailleerde bestudering van de gang van zaken bij de chronische transplantaat afstoting mogelijk te maken, wordt geconcludeerd dat er gezocht moet worden naar een hiertoe geëigend model van orthotope harttransplantatie. In de Paragraaf 1.2 wordt aangetoond dat bij voorkeur een dergelijk model niet beïnvloed dient te worden door de nadelige bijwerkingen als gevolg van de toediening van immunosuppressiva. Tevens wordt besproken dat het gebruik van homoloog donor bloed tijdens de transplantatie procedure van invloed kan zijn op de prognose van het transplantaat en daarom van belang geacht moet worden bij de beoordeling van de resultaten.
In Paragraaf 1.3 worden de doelstellingen van dit onderzoek als volgt geformuleerd:
De eerste doelstelling is geweest het ontwikkelen van een model van chronische afstoting van een orthotoop harttransplantaat bij de hond door gebruik te maken van DLA-identieke beagles uit hetzelfde nest en zonder de toepassing van immunosuppressiva.
De tweede doelstelling is geweest de mogelijkheid te onderzoeken of een orthotope harttransplantatie uitgevoerd kan worden zonder dat hierbij gebruik gemaakt wordt van homoloog donor bloed.
In het Tweede Hoofdstuk zijn de gegevens vastgelegd met betrekking tot het gebruikte materiaal en de gevolgde methoden.

De resultaten van dit onderzoek zijn gedocumenteerd in het Derde Hoofdstuk. Uit deze gegevens blijkt dat na orthotope harttransplantatie en zonder de toepassing van immunosuppressiva, identiciteit voor het DLA histocompatibiliteits complex tussen donor en ontvanger bij beagles afkomstig uit hetzelfde nest, resulteert in het kenmerkende histologische beeld van de chronische transplantaat afstoting, welke wordt aangeduid als chronische obliterative arteritis.
In paragraaf 3.2. wordt aangetoond dat het technisch mogelijk is een orthotope harttransplantatie bij de hond uit te voeren zonder daarbij gebruik te maken van homoloog donor bloed.
In het eerste deel van Hoofdstuk Vier wordt het verkregen model van de chronische afstoting van een orthotoop harttransplantaat bij de hond besproken en worden een aantal mogelijkheden voor verder onderzoek met behulp van dit model vermeld.
Het tweede deel van Hoofdstuk Vier bevat een meer uitgebreide discussie over de toepassing van hemodilutie en autotransfusie bij de orthotope harttransplantatie, met name bij kleine honden.
Na een korte bespreking van het verband tussen de toediening van bloedtransfusies en transplantatie, worden tenslotte de levensduur van de 10 honden na transplantatie uit het tweede experiment vergeleken met de levensduur na transplantatie van 13 honden welke in het verleden door hetzelfde chirurgische team en onder dezelfde omstandigheden werden geopereerd, maar met het gebruik van homoloog donor bloed.
Op grond van deze vergelijking is het zeer waarschijnlijk dat de toediening van homoloog donor bloed tijdens de transplantatie procedure van invloed is op de prognose van het harttransplantaat.

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