A STUDY OF ALLOGRAFT KIDNEY REJECTION OCCURRING SIMULTANEOUSLY IN WHOLE ORGAN AND EAR CHAMBER GRAFTS IN THE RABBIT

BY J. B. HOBBS* AND W. J. CLIFF

(From the Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia)

(Received for publication 31 October 1972)

Most of the early studies on allograft rejection have been concerned with the morphological changes seen either in biopsy tissue or after removal of whole organs at different time intervals after grafting. Some have studied the organs in situ by indirect techniques which monitored physiological capacity or alterations in blood flow characteristics.

Various tissues have been studied microscopically by heterotopic implantation of organ fragments into such sites as brain (1), anterior chamber of the eye (2), renal capsule (3), hamster cheek pouch (4), and the transparent ear chamber of the rabbit (5). However, when these techniques were applied to allografted tissues, few were successful; and all those that did yield a clear microscopic view of the functioning tissues in vivo, such as hamster cheek pouch, the anterior chamber of the eye, or the rabbit ear chamber, exhibited atypical responses which were quite unlike the rejection that occurred when the same tissues were transferred as whole organs.

When pieces of kidney were allografted into the rabbit ear chamber, they reestablished their blood circulation in 2-4 days by anastomosis of their cut vessels to the adjacent vasculature of the ear chamber membrane (6). Autografts appeared to function indefinitely, since autologous renal tissue was observed in the rabbit ear chamber without change for 6 mo after fixation, and functioning myometrial autografts were observed for periods of up to 1 yr (6). Allografted renal tissues in the rabbit ear chamber reject after periods of up to 3 mo (6). A whole kidney allografted in similar rabbits is rejected in 7 days. When a whole kidney is allografted with an established ear chamber allograft from the same donor already in situ, both grafts reject synchronously over the following 7 days.

This report describes the changes seen in the unmodified rejection of renal allografts occurring simultaneously in whole organs and minute implants within transparent ear chambers. Microscopic details of the process which results in whole organ renal allograft destruction can thus be studied in vivo by observation of the ear chamber grafts which are being rejected synchronously with the whole organ.

* Present address: Department of Medicine, University of Melbourne, Parkville, Victoria, Australia.

776 THE JOURNAL OF EXPERIMENTAL MEDICINE • VOLUME 137, 1973
Materials and Methods

Rabbits.—Recipient rabbits were chosen from a closed colony of half-lap rabbits maintained at the Australian National University. Donor rabbits were unrelated; they were either from the same colony or sometimes from an entirely different strain from outside this colony.

Grafting Procedures:—

Ear chamber grafts: The procedure for transparent ear chamber insertion and the grafting of organ fragments into them was as previously described (6). The ear chamber grafts were taken from the donor’s right kidney through a small flank incision. A wedge of cortex approximately 0.5 X 0.8 cm was transferred to the stage of a dissecting microscope, and the fragments for ear chamber implantation were prepared from this by a second operator. A wedge of Gelfoam (Upjohn Co., Kalamazoo, Mich.) placed in the renal incision provided swift hemostasis, and the flank incision was closed with continuous 3/0 chronic gut sutures (Ethicon, Inc., Somerville, N. J.).

As soon as these grafts had their circulation reestablished, within 3 or 4 days of the first procedure, a second operation was performed to transfer the whole left kidney from the same donor to the same recipient. One operator removed the left kidney from the donor through a broad abdominal incision, while a second readied the site in the recipient rabbit by removing its left kidney, and preparing the renal vessels transected close to the excised kidney for anastomosis to the graft vessels.

Whole renal grafts: Whole renal grafts into the abdomen were performed by an adaptation of the method described by Owen (7). Sometimes the whole organ was grafted into the recipient’s neck, anastomosing the renal artery end-to-end with the internal carotid and the vein end-to-side to the external jugular vein. The arterial anastomosis was effected by six to eight interrupted sutures of 9.0 nylon (Ethicon), both inserted with a microvascular needle holder using X 4 magnified vision (Keeler telescopic spectacles, Keeler, London, England). Ischemia time for the transplanted kidney was 45 rain-1 h. The kidney was held in place by two to three sutures through the capsule. Transplants in either site had a polythene tube 2 mm in diameter tied into their ureters by a double ligature of 5.0 braided silk (Ethicon) to act as a catheter. The other end of the catheter tube was passed under the skin to a point just behind and between the ears where it was brought out and fixed with a 3.0 silk suture (Ethicon) through the skin. This ensured that the rabbit did not pull the catheter out, and periodic collections of urine could be made by sample bottles attached to the ear. When the graft was placed in the neck, it allowed more direct access for palpation and biopsy. When the allograft was placed in the neck, the recipient rabbit retained both its own kidneys, and when it was grafted into the abdomen, it replaced the left kidney, the right kidney of the recipient remaining in situ.

Photomicroscopy.—Photographic observations on the ear chamber grafts and urinary cellular contents were made with a 35 mm Leitz camera and a 16 mm cine camera (Vinten Ltd., London, England) at both normal speed (16 frames/s) and time-lapse (60 frames/min to 4 frames/min).

Whole Organ Graft Observation.—Urine was collected at 2-4 h intervals and observed microscopically without previous centrifugation. Urinary cell numbers and types were recorded, and samples were stored frozen for gel electrophoresis.

Daily needle biopsies were performed on the grafted organ, under mild anesthesia (Nembutal, 0.3 ml/kg in 10 ml saline) with a Franklin-Silverman biopsy needle designed for pediatric use. The core of extracted tissue was immediately fixed as described below for Durcupan embedding. When the whole organ was placed in the neck, it was possible to make daily measurements of the long and short axis of the kidney with calipers through the skin.

Observation of the Recipient Rabbit.—A daily clinical assessment of the rabbit’s condition was made and its blood pressure was recorded at least daily with a Grant’s capsule on the ear.
artery. A peripheral leukocyte count and differential as well as erythrocyte sedimentation rate (ESR)\(^1\) and hematocrit determination were also made daily.

**Fixation, Embedding, Sectioning, Staining, and Viewing of Tissues.**—The ear chambers and needle biopsies were fixed in 4% glutaraldehyde in cacodylate buffer at pH 7.2, 4°C, for 1 h (8). Whole organs being removed were fixed *in situ* by intra-arterial perfusion of the organ with Millonig’s modification of Karnovsky’s fixative (9, 10) at room temperature, under a pressure equal to the intraluminal pressure recorded at the time of insertion of the perfusing cannula proximal to the arterial anastomosis. After perfusion for 2 h, the organ was cut lengthwise and blocks trimmed under a dissecting microscope from the cut face.

Blocks from both methods of fixation were then allowed to soak thoroughly in buffer before being transferred to buffered osmium fixative (11) for 2 h at 4°C, then to uranyl acetate (1% in water) for 1 h at bench temperature. After dehydration with acetone, the tissues were embedded in Durcupan (Fluka AG., Basel, Switzerland).

Sections approximately 1 μm thick were cut with glass knives and stained with Richardson’s stain (12) for light microscopy. Sections for ultrastructural examination were cut with a diamond knife (E. I. Dupont de Nemours & Co., Wilmington, Del.), stained with alkaline lead citrate solution (13), and viewed with a Siemens Elmiskop electron microscope (Siemens Corp., Iseling, N. J.).

### RESULTS

A total of six rabbits had simultaneous ear chamber and whole organ allografts (Table I). On the first 2 days after whole organ allografting, clear urine flows from the catheter in the transplanted ureter, and microscopically this is usually clear or contains only a few erythrocytes or tubular epithelial cells. There is no change in the ear chamber grafts. (Fig. 1 A).

**At 48 h.**—The whole organ is still normal to palpation and the urinalysis is normal. The ear chamber grafts begin to show large fluctuations in their blood flow (Figs. 1 and 2). Most of the time there is increased velocity and volume of blood flow which alternates irregularly and sometimes abruptly, at intervals of from 10 min to ½ h, with periods which generally last from 1 to 5 min of greatly decreased general blood flow. The ear chamber host vessels appear normal (Fig. 1). At the same time, the veins and venules of the grafts develop dilations which alternate along their lengths with short constricted regions (Figs. 1 and 2). The diameters of the dilated regions may be up to 20 times that of the constricted regions of the same vessels. The small graft arterioles, such as afferent and efferent glomerular arterioles, show similar, though less pronounced, patterns or irregular dilatation and constriction along their lengths (Fig. 2 A). The larger graft arteries, such as interlobulars, remain uniform along their lengths. However, these larger arteries constrict and dilate as the blood flow varies within the grafts, as described above. Even when the blood is flowing constantly at a high rate, time-lapse cinemicroscopy of such arteries reveals a regular rhythmic constriction and dilatation, which is moderate in degree and uniform along its length, with a periodicity of once every 5 min. This cannot be appreciated by direct observation.

\(^1\) Abbreviations used in this paper: ESR, erythrocyte sedimentation rate.
| Rabbit | Ear chamber observations | Days post-operatiom | Whole organ graft site | Whole organ graft face | Recipient rabbit face | Terminated at removal of whole organ day? | Fixed in formalin for transmission of rabid | Terminated 14 days post-transplantation | Allowed to recover | Removed and perfused with Karnovsky fixative day? | Removed day/s and Fixative Karnovsky fixative and removed day? | Removed day/s and Fixative Karnovsky fixative and removed day? | Rabbit moribund and died at 14 days post-transplantation | Terminated at 16 days post-transplantation | Terminated 14 days post-transplantation | Terminated at 14 days post-transplantation |
|--------|--------------------------|---------------------|-----------------------|-----------------------|----------------------|-----------------------------------------|---------------------------------------------|-------------------------------------------|---------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|-------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|
| Aa1    | Nil                      | 2                   | Ear chamber instilled | Nil                   | Aa1                  | 2                                      | Aa1                                        | Terminated at removal of whole organ day? | Fixed in formalin for transmission of rabid | Terminated at removal of whole organ day? | Removed and perfused with Karnovsky fixative day? | Removed day/s and Fixative Karnovsky fixative and removed day? | Removed day/s and Fixative Karnovsky fixative and removed day? | Terminated at 16 days post-transplantation | Terminated at 16 days post-transplantation | Terminated at 14 days post-transplantation | Terminated at 14 days post-transplantation |
RENAL REJECTION IN VIVO
At the same time numerous platelets are seen adhering to the graft blood vessel endothelium both in regions which are otherwise normal and also in regions where irregular dilatations and constrictions are present. This platelet adhesion may be seen occurring in the presence or absence of leukocyte adhesion to the vessel endothelium. In this highly characteristic form of platelet adhesion the platelet is fixed by one pole to the endothelium and its free end oscillates in the flowing bloodstream (Figs. 3–5). When flow pauses momentarily, the platelet assumes a position perpendicular to the vessel wall. As many as 20 platelets may be seen adhering individually to the endothelium in this way in one high-power field. Most of the platelets thus observed appear optically denser and larger, and have blunter ends than unattached platelets. Such adherent platelets usually remain firmly attached and have been observed thus for several hours. If such a platelet becomes detached, it is usually replaced within seconds by another platelet or at times by a leukocyte, which adheres apparently to precisely the same point on the endothelium.

Shortly afterwards leukocytes begin to adhere to the swollen venular endothelium, about three to four per high-power field (Figs. 4 and 6). These leukocytes are generally nongranular and vary widely in size from that of small lymphocytes (8 μm) up to four to five times that diameter. The majority lie in the middle of the range (≈25 μm). Time-lapse cinematography indicates that these cells move very slowly when compared with the occasional polymorphs or eosinophils seen adhering in the same fields.

Platelets also adhere progressively to the leukocytes which are sticking to the vessel walls (Fig. 6). Up to 10 platelets may be seen adhering to one leukocyte, producing a pincushion or porcupine effect (see Fig. 5 F). If one such leukocyte is not firmly attached, and commences rolling along the wall, platelets can be seen adhering by one pole to the cell, and by the other to the endothelium on the side away from progression (Fig. 5 E). Subsequently, platelets can also be seen adhering individually by their flat surfaces to both endothelium and adherent leukocytes. Aggregation of platelets into platelet clumps is not common, and when it does occur, usually one adheres to the endothelium by one pole as described above, then three or four others will attach themselves to it, lying

Fig. 1. Three views of a transparent ear chamber membrane showing the changes observed in vivo in early rejection. (A) Three allografts (G) well established. The vessels on the periphery of the membrane (RV) are of recipient origin. Both they and the graft vessels have good blood flow and the vessel walls are parallel. The arteriole to most of the ear chamber (A) has been completely converted to supply the renal grafts. × 10. (B) The same membrane and grafts showing the earliest detectable stage of rejection. The graft vasculature is irregularly dilated and constricted. The recipient vessels (A and RV) are slightly dilated, reflecting the increased blood flow through the graft, but have uniform lumina. × 10. (C) The same membrane and grafts several hours after Fig. 1 B. The graft vasculature is much more dilated, with irregular areas of constriction. Note uniformity of the recipient vessel walls (A and RV). × 10.
parallel in the fashion of a shingle roof (Fig. 5 D) but usually only the original
one is attached to the wall. True thrombus formation is very rare, but has been
observed. Towards the latter part of the period from 48 to 72 h, the irregularity
of the graft vessels becomes more accentuated so that the diameter of dilated
portions of the veins may be up to 30 times that of the constricted portions of

Fig. 3. A platelet (circled) adhering to the graft endothelium as an individual unit by one
pole, in the absence of leukocyte adhesion. These pictures were taken within minutes of each
other, and illustrate the movement of the unattached pole in the bloodstream. × 1,400.

Fig. 4. A platelet (circled) adhering to the graft endothelium after early nongranular
leukocytic adhesion has commenced (arrows). × 1,400.

Fig. 2. (A) In vivo photographs showing the detail of the irregular constrictions (arrows)
and dilatations seen in an artery from the graft shown in Fig. 1 B. × 1,200. (B) Detail of the
irregular vessels seen elsewhere in the same graft as Fig. 1 B at the same time. × 300. (C) The
extreme dilatation of the graft vessels at the stage of rejection shown in Fig. 1 C. There is
accentuation of laminar patterns in the blood flowing through these grossly dilated areas.
× 300.
Fig. 5. Diagrammatic representation of the forms of platelet adhesion observed during early rejection. A, B, and C illustrate the oscillating platelet as seen in Figs. 3 A, B, and C. Fig. 5 D shows the form that the rare platelet aggregation took. Fig. 5 E shows the form of platelet adhesion to both endothelium and leukocytes after leukocytic adhesion commenced. Fig. 5 F shows the form of multiple platelet adhesion to an adherent leukocyte. Each is attached as a single unit by one pole and the other pole is free to move independently of the others.

Fig. 6. Platelets (circled) adhering to leukocytes (arrows) which are accumulating along the graft endothelium. \( \times 1,400 \).
the same vein. The dilated walls are barely visible and where it is possible to see them in vivo they have the appearance of single layers of plump endothelium. Even though the blood is still flowing briskly, it can be readily seen that many of the erythrocytes are becoming aggregated into irregular clumps, and that more than the usual number of single erythrocytes are poikilocytes. These erythrocyte aggregates are also seen in the ear chamber host vessels. At the same time, the peripheral blood ESR rises abruptly. Toward the end of this period, the finer details of the grafts become clouded by developing edema. Large numbers of lymphocytes can be seen within the tubular lumen drifting towards the collecting ducts. One or two microhemorrhages consisting of from 10 to 50 erythrocytes can be seen within the graft parenchyma in the succeeding few hours, apparently occurring from capillaries. Some graft vessels develop intermittent stasis.

A needle biopsy of the whole organ at this stage shows some ultrastructural vacuolation of the tubular cells. The endothelium of intertubular capillaries appears well preserved but some have pointed luminal margins. Occasional lymphoid cells containing numerous cytoplasmic polyribosomes can be seen in contact with endothelial cells. Platelets in very close proximity to endothelium show vacuolation and loss of some or all α-granules and very dense granules (Fig. 7).

The Period 72–96 h.—The whole organ is now approximately double its original size on palpation. Urine flow is reduced, in one case to 1 ml/h. Microscopically the uncentrifuged urine has 5 leukocytes and 20 erythrocytes per high power field. When the urine is examined fresh from the catheter, fibrin strands are seen forming on the slide. The ESR continues to rise (see Fig. 8).

The ear chamber grafts show more extensive nongranular and occasional granular leukocytic adhesion in the graft venules and occasional leukocytes adhering in the arterioles. Stasis becomes more pronounced, the microhemorrhages enlarge in the graft tissues, and hemorrhages into Bowman's space can also be observed. Leukocytes are discernible in the graft tissue but their detail is not clear. Platelets are observed adhering as before and nearly all adherent leukocytes have platelets attached. The erythrocytes in both the ear chamber vessels of host origin and graft vessels are now very clumped. The host vessels of the ear chamber show no reaction except those “downstream” from anastomoses with graft veins which show leukocytic rolling but not adhesion.

The needle biopsies of the whole organs examined by electron microscopy show increased cytoplasmic vesiculation of tubular epithelial cells. The endothelial cells are swollen in most vessels and numerous lymphoid cells rich in ribosomes are in contact with their luminal surface. Within the whole graft and ear chamber graft tissue, large numbers of lymphocytes and some polymorphs are seen together with hemorrhage in the graft tissues, particularly in regions adjacent to blood vessels.
The Period 96–120 h.—Urine output from the transplanted whole kidney has almost stopped. The peripheral blood has a rising leukocyte count (Fig. 8) and the ESR is 40–50%. The ear chamber host and graft vessels both have extreme clumping of the contained erythrocytes and the lumina of graft vessels of all sizes are greatly narrowed by adherent leukocytes, so much so that some are completely filled with leukocytes and no blood flows through them. There is a

![Graphs showing changes in blood pressure, hematocrit, ESR, and peripheral blood leukocyte count over 7 days.](image)

Fig. 8. The relative changes in blood pressure, hematocrit, ESR, and peripheral blood leukocyte count of three recipient rabbits in the days after both ear chamber and whole renal allografting procedures.

Fig. 7. (A) An electron micrograph of a blood vessel in a renal allograft fixed in the earliest stage of rejection when platelet adhesion was first noticed. The single platelet adjacent to the graft endothelium (GE) shows extensive dilatation of the surface-connected tubular system (open arrows) and a comparative lack of granules. Two of these (filled arrows) appear to be on the point of discharge from the platelet. Note absence of pseudopodia. × 61,000. (B) An electron micrograph of a similar platelet in the vessel of a rejecting ear chamber allograft. Here there is pronounced dilatation of the surface-connected tubular system (open arrows), with apparent absence of granules. It is particularly noteworthy that the platelet has retained its disk shape, with good preservation of the microtubules and no pseudopodia formation. The adjacent graft endothelium appears well preserved. × 25,000. (C) The form of the platelets present in the ear chamber (recipient vessels) under the same conditions as Figs. 7 A and B. There is good preservation of the platelet granules, even when adjacent to the recipient endothelium (RE). × 22,000. Inset, × 6,000.
rapid diminution of recognizable renal parenchyma remaining and the micro-
hemorrhages are extending and coalescing to form confluent areas of hemor-
rhage limited to the graft tissues. Some collecting ducts remain and begin to
form cystic spaces. The epithelial cells lining these spaces at times develop cilia
which beat vigorously in vivo and after fixation have a typical 9 + 2 ultra-
structural appearance. Even within the hemorrhagic areas, large numbers of
leukocytes can be seen within the renal parenchyma. Within the vessels plate-
lets can still be observed adhering to leukocytes.

A needle biopsy of the whole organ shows a progression of the leukocytic
infiltration of the tissues seen in the previous period, with extensive deteriora-
tion of renal tubular epithelial cells. Glomeruli have some swelling of the epil-
thelium of Bowman’s space and occasional leukocytes within tuft capillaries.
In some areas endothelial cells appear to have separated from their glomerular
basement membrane. For the most part, however, the glomeruli are relatively
well preserved.

The Period 120-144 h.—The host rabbit’s blood pressure rises abruptly to
almost double its previous systolic level (Fig. 8) and urine flow from the whole
organ has almost ceased. In the ear chamber the graft parenchyma is very
difficult to identify and few tubules can be seen. Blood flows in only a few of the
organ capillaries and then only as an erratic trickle through an irregular channel
between rows of adherent, mainly nongranular leukocytes three to four deep on
the endothelium. Platelets continue to adhere to these leukocytes in large
numbers as described previously. Most of the glomeruli in the ear chamber
grafts are now bloodless apparently because of lack of afferent blood supply.
However a few glomeruli in stasis have their capillaries distended with erythro-
cytes. Electron microscopy of a needle biopsy of the whole organ at this stage
shows very few recognizable tubules. The bulk of the tissue is made up of inter-
stitial lymphoid cells, with some polymorphs and hemorrhage.

The Period 144-168 h.—The recipient rabbits are now quite ill. One died on
this day of a perforated gastric ulcer with hematemesis (Table I). The whole
bloodstream is coarsely granular and microscopically composed of large masses
of aggregated erythrocytes, about 250 µm in greatest diameter. Blood pressure
is now up to 165 mm Hg systolic, double the normal resting value (Fig. 8). The
ear chamber grafts have stasis of all vessels and no recognizable graft paren-
chyma remains.

The whole organ is perfused with Karnovsky fixative as described above, and
in one case the animal was allowed to recover (Table I). Sections from paraaffin-
embedded tissues show relative sparing of the glomeruli which, however, con-
tain occasional polymorphonuclear leukocytes and lymphocytes. There is
extensive tubular atrophy and the urinary space from Bowman’s space to the
collecting tubules contains eosinophilic material and clear vacuoles suggesting
lipid. The surviving tubular epithelial cells are very swollen. There is extensive
and confluent infiltration of the renal tissue by large lymphoid cells which form
cuffs that are widest around the large arteries and taper in width with the size of vessels, almost disappearing just where the afferent arterioles join the glomeruli (Fig. 9). Several large arteries show leukocytes in their walls (Fig. 9).

**DISCUSSION**

In the absence of a simultaneous allograft stimulus using tissue from the same donor, ear chamber allografts from adult donors routinely survive for several months, without modification of the rejection process (6). The presence of whole organ grafts, like that of skin grafts or intraperitoneal injections of spleen cells obtained from the same donor, triggers a more rapid rejection of the ear chamber grafts (6), which is completed within 7 days of the grafting of the whole kidney.

The correspondence of in vivo microscopic observation in the ear chamber grafts to the histological preparations of serial needle biopsies of the whole organ establishes that the process of rejection in the whole organ can be monitored in the ear chamber grafts. Using this method, it is clear that changes can be observed in vivo principally in the graft vessels long before early histological evidence of rejection is present in biopsies of the whole organ. Graft vascular constrictions and dilatations, varying patterns of blood flow, and adhesion of individual platelets without aggregation of platelets, behavior occurring at about 48 h accompanied by mild nongranular leukocytic adhesion, is the earliest evidence of rejection observed in vivo. In the following period (72–96 h) when the usual criteria of whole organ rejection first appear, such as increasing organ size, rising ESR, and reduced volume of urine which contains both protein and leukocytes, biopsy of the whole organ shows platelet and leukocytic adherence to the graft endothelium.

The ear chamber grafts reflect these changes in vivo. The rising ESR is paralleled by the increasing aggregation of erythrocytes observed in both graft and recipient vessels and the hematuria by the presence of erythrocytes in Bowman’s space of the ear chamber graft glomeruli. The drop in urinary output is reflected in the in vivo observation of increasing irregularity of graft blood flow and intermittent stasis. The parenchymal destruction seen in whole organ biopsies in the period 96–120 h is reflected in the ear chamber grafts by a diminution in the number of recognizable tubules and an extension of the microhemorrhages. It is interesting that the collecting ducts are relatively resistant to this process and many commence to dilate into cystic spaces. Since embryologically they are derived from the ureteric bud and not the metanephros (14, 15) it could be that, although ureters do reject (16), they may evoke a less severe rejection process.

Glomeruli have a relatively intact structure in biopsies of the rejecting whole organ and this corresponds to the in vivo observation that, although glomeruli of the ear chamber graft have little blood flow through them, they are clearly visible, and it is rare to find leukocytes in them either by direct observation or
by time-lapse cinemicroscopy. The continuing increase in ESR reflects the progression of intravascular erythrocyte agglutination observed in the blood vessels of both graft and donor origin in the ear chambers. In the period 120–144 h, the circulatory stasis observed in the ear chamber graft vessels and the progressive obstruction of their lumina by leukocytes is reflected by the great reduction in urine being produced by the whole organ and agrees with the appearance of the needle biopsy.

For some time there has been increasing emphasis on the role of graft vessel damage in allograft destruction (17–31). The dramatic changes in vessel form and function described above, occurring when the platelet adhesion is developing in the earliest stages of rejection, suggest that these adhesive platelets play some role in the rejection process. Porter et al. (18) drew attention to intravascular platelet aggregates in human kidney allografts, and others, using sensitized animals (32–34), have commented on the possible role of platelets in canine renal rejection. Platelets have also been suggested as pathogenic factors in rapid human allograft rejection (35). Recently Busch et al. (31), describing the vascular lesions in early human renal rejection, commented on the possibility of platelets producing vessel damage without necessarily forming thrombi.

Apart from some of the early descriptions of the platelet (36–38) and some more modern studies in vivo (39, 40), by far the bulk of knowledge concerning the properties of the platelets has come from in vitro studies. These have revealed that under various stimuli resulting in aggregation, platelets may release some or all of their pharmacological contents which include histamine (41), serotonin (42, 43), ATP (44), ADP (45), fibrinogen (46), adrenaline (47), nor-adrenaline (48), lysosomes (49), acid phosphatase (49), elastase (50), and a so-far unidentified heat-stable factor, of mol wt less than 10,000, which causes increased vascular permeability (51, 52).

The morphological changes observed in vivo and reported here to occur in the adherent platelets, such as darkening and loss of refractibility, are similar to those platelet changes observed by Bizzozero (36, 37) and others (38, 40) in vivo in the course of producing hemostasis and viscous metamorphosis during which platelets undergo the release reaction (53). Further, platelets which have undergone the release reaction in vitro (54–57) are very similar in ultrastructural appearance to the platelets in graft vessels described in this report.

Fig. 9. Photograph showing a section from the whole renal allograft removed at day 6. The larger area shows the cuffing of lymphoid cells widest around the larger vessels, and tapering proportionately with the diameter of a branch which supplies a glomerulus. The perivascular cuff almost disappears just where the afferent arteriole enters the glomerular tuft. The open arrow indicates several lymphoid cells present within the vessel wall many of which are pyroninophilic. The tubular epithelium is swollen and vacuolated, but the glomerulus is relatively well preserved. Karnovsky fixative, paraffin embedded, hematoxylin and eosin stain. × 1,200. Inset A shows detail of a larger vessel in the same section with several cells, one resembling a neutrophil in its wall (open arrows). × 1,200. Inset B indicates the evenness of the perivascular infiltrate in another area from the same specimen. × 300.
Evidence has accumulated dramatically that platelets can release their contents under the influence of immune complexes (58–62) and by interaction of the platelet with antigenically activated leukocytes (53, 63–66). This striking similarity with the circumstances under which we observed platelet adherence described in this report becomes even closer to Henson’s report that there was no lysis of the platelets during the in vitro release of their vasoactive amines (55). The identity of the leukocytes involved in these studies is disputed. Henson suggests that they are monocytes (63) but also believes neutrophils can behave in this way (55), and Barbaro and Schoenbechler felt that the small lymphocytes were responsible (64); but Aikawa takes the view that several cell types may be responsible (54). Aikawa’s (54) ultrastructural studies showed pseudopodia of the adherent platelet in vitro inserted into the interacting leukocyte cytoplasm, with discharge of platelet granules. The resultant ultrastructural reduction of granules in adherent platelets resembled the appearance of platelets within graft vessels described in the electron micrographs in this report (Fig. 7).

There is little doubt that, once liberated, platelet constituents have the capacity to alter vessels (51, 67–72) and may well account for the graft vasoactivity and vessel destruction described in this report. Cochrane and Hawkins (73, 74) have postulated a similar role for platelets in the vasculitis occurring in the Arthus reaction to that occurring in anaphylaxis or serum sickness (75–78). They felt that histamine released from platelets is responsible for increased permeability, which may then allow implantation of circulating immune complexes into vessel walls and thus provoke a vasculitis (73, 74). Horowitz et al. (79) have demonstrated in canine renal allografts that there is deposition of immune complexes within the walls of the larger graft arteries such as the interlobular and afferent arterioles by the 3rd day. This is about the time we observe the vascular reaction in similar vessels in rabbit allografts and could result from the observed adherent platelet release reaction. Hughes and Tonks (80) have shown that platelet aggregation can produce vasculitis in the myocardial circulation, and others have shown that platelet aggregation by antigen-antibody complexes, viruses, bacteria, and endotoxin can cause blood vessel damage (52, 81–84) and focal lesions in the rabbit aorta (76).

From this it is clear that agents with proven vasoactivity and others with at least a potential for tissue destruction may be liberated from platelets under circumstances similar to those described in this report at the earliest evidence of rejection in vivo. This is strong evidence that platelets may play a large part in pathogenesis of diseases manifesting vascular damage without forming aggregates or taking part in thrombosis. Furthermore, in the earliest stages of unmodified rejection, the platelets may play several roles in the destructive process which results in vascular derangement and subsequent graft elimination.
SUMMARY

When portions of adult renal tissue are allografted into the rabbit ear chamber, they usually survive for periods of up to several months (6). When a kidney from the same donor is grafted as a whole organ, the ear chamber grafts then reject with the whole organ in 7 days. During that time serial needle biopsies of the whole organ are compared with the in vivo appearance of the ear chamber grafts. This establishes that the changes occurring in the ear chamber grafts are monitoring the rejection process proceeding in the whole organ grafts.

Dramatic vascular changes herald the earliest stages of unmodified rejection. A highly characteristic form of individual discrete platelet adhesion to both endothelium and adherent leukocytes is observed which is associated with the release reaction. At times as many as 20 such discrete platelets are clearly visible in profile in one high-power field. This demonstrates in vivo a mechanism whereby vascular and parenchymal damage may be produced by platelet contents, without previous aggregation or thrombus formation being necessary.

REFERENCES

1. Willis, R. A. 1935. Experiments on the intracerebral implantation of embryo tissues in rats. Proc. R. Soc. Lond. B Biol. Sci. 117:400.
2. Greene, H. S. N. 1943. The heterologous transplantation of embryonic mammalian tissues. Cancer Res. 3:809.
3. Wheeler, H. B., J. M. Corson, and G. J. Dammin. 1966. Transplantation of tissue slices in mice. Ann. N. Y. Acad. Sci. 129:118.
4. Cohen, S. N. 1961. Comparison of autologous, homologous, and heterologous normal skin grafts in the hamster cheek pouch. Proc. Soc. Exp. Biol. Med. 106:677.
5. Williams, R. G. 1954. Microscopic studies in living mammals with transparent chamber methods. Int. Rev. Cytol. 111:339.
6. Hobbs, J. B., and W. J. Cliff. 1971. Observations on tissue grafts established in rabbit ear chambers: a combined light and electron microscopic study. J. Exp. Med. 134:963.
7. Owen, E. R. 1969. Microtechniques in organ transplantation. In Recent Advances in Paediatric Surgery. A. W. Wilkinson, editor. J & A Churchill Ltd., London. 2nd edition. 251.
8. Sabatini, D. D., K. Bensch, and R. J. Barrnet. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructural and enzymic activity by aldehyde fixation. J. Cell Biol. 17:19.
9. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27:137a. (Abstr.)
10. Millonig, G., and M. Bosco. 1967. Some remarks on paraformaldehyde as fixative. J. Cell Biol. 35:177a. (Abstr.)
11. Caulfield, J. B. 1957. Effects of varying the vehicle for OsO4 in tissue fixation. J. Biophys. Biochem. Cytol. 3:827.
12. Richardson, K. C., L. Jarett, and E. H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* 35:313.

13. Venable, J. H., and R. Coggshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25:407.

14. Kissane, J. M. 1966. Development of the kidney. In *Pathology of the Kidney.* R. H. Heptinstall, editor. Little, Brown and Company, Boston.

15. Torrey, T. W. 1965. Morphogenesis of the vertebrate kidney. In *Organogenesis.* R. L. DeHaan and H. Ursprung, editors. Holt, Rinehart and Winston, Inc., New York. 559-579.

16. Paccone, F., A. Aboul-Enein, T. Shikata, and W. J. Dempster. 1965. Changes in the transplanted ureter. *Br. J. Exp. Pathol.* 46:519.

17. Altman, B. 1963. Tissue transplantation: circulating antibody in the homotransplantation of kidney and skin. *Ann. R. Coll. Surg. Engl.* 33:79.

18. Porter, K. A., R. Y. Calne, and C. F. Zuckosi. 1964. Vascular and other changes in 200 canine renal homo transplants treated with immunosuppressive drugs. *Lab. Invest.* 13:809.

19. Murray, J. E., A. G. Ross Shell, R. Moseley, R. Knight, J. D. McGavic, and G. J. Dammin. 1964. Analysis of mechanism of immunosuppressive drugs in renal homotransplantation. *Ann. Surg.* 160:449.

20. Horowitz, R. E., L. Burrows, F. Paronetto, D. Dreiling, and A. E. Kark. 1965. Immunological observations on homografts. II. The canine kidney. *Transplantation.* 3:318.

21. Dammin, G. J. 1966. Renal transplants: correlation of histologic pattern with function. The Kidney. *Int. Acad. Pathol. Monogr.* 6:445.

22. Dempster, W. J., C. V. Harrison, and R. Shackman. 1964. Rejection processes in human homotransplanted kidneys. *Br. Med. J.* 2:969.

23. Hamburger, J., J. Crosnier, and J. Dormont. 1965. Experience with 45 renal homotransplantations in man. *Lancet.* 1:983.

24. Kincaid-Smith, P. 1964. Vascular changes in homotransplants. *Br. Med. J.* 1:178.

25. Kincaid-Smith, P. 1967. Histological diagnosis of rejection in renal homografts in man. *Lancet.* 2:849.

26. Porter, K. A., K. Owen, J. F. Mowbray, W. B. Thomson, J. R. Kenyon, and W. S. Peart. 1962. Obliterative vascular changes in four human kidney homotransplants. *Br. Med. J.* 2:639.

27. Porter, K. A., T. L. Marchioro, and T. E. Starzl. 1965. Pathological changes in 37 human renal homotransplants treated with immuno-suppressive drugs. *Br. J. Urol.* 37:250.

28. Lewis, D. H., S. E. Bergentz, U. Brunius, and L. E. Gelin. 1968. Blood flow in kidney transplants. Studies at exploration of a previous rejected kidney, with special reference to rejection. *Ann. Surg.* 168:803.

29. Dougherty, J. C., M. Hollenberg, R. Minick, J. C. Whitsell, and F. Veith. 1969. Intrarenal distribution of blood flow in dog renal allografts. *Ann. Surg.* 169:560.

30. Trentin, J. J. 1966. The arterial obliteratorive lesions of human renal homografts. *Ann. N. Y. Acad. Sci.* 129:554.

31. Busch, G. J., E. S. Reynolds, E. G. Galvanek, W. E. Braun, and G. J. Dammin.
1971. Human renal allografts. The role of vascular injury in early graft failure. *Medicine (Baltimore)*. 50:29.

32. Lowenhaupt, R., and P. Nathan. 1967. Variation in blood platelet counts during rejection of dog kidney allotransplants. *Fed. Proc.* 27:442.

33. Lowenhaupt, R., and P. Nathan. 1969. The participation of platelets in the rejection of dog kidney allotransplants: hematologic and electron microscopic studies. *Transplant. Proc.* 1:305.

34. Macdonald, A., G. J. Busch, J. L. Alexander, E. A. Pheteplace, J. Menzoain, and J. R. Murray. 1970. Heparin and aspirin in the treatment of hyperacute rejection of renal allografts in presensitized dogs. *Transplantation.* 9:1.

35. Starzl, T. E., H. J. Bochmig, H. Amemiya, C. B. Wilson, F. J. Dixon, G. R. Giles, K. M. Simpson, and C. G. Halgrimson. 1970. Clotting changes, including disseminated intravascular coagulation during rapid renal-homograft rejection. *N. Engl. J. Med.* 283:383.

36. Bizzozero, G. 1882. Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* 90:261.

37. Bizzozero, G. 1882. Sur un novel element morphologique du sang. Chez les mammifers vivants. Et sur son importance dans la thrombose et dans la coagulation. *Arch. Ital. Biol.* 1:1.

38. Eberth, J. C., and C. Schimmelbusch. 1886. Experimentalle Untersuchungen uber Thrombose. *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* 103:39.

39. Sandison, J. C. 1931. Observations on the circulating blood cells, adventitial (Rouget) and muscle cells, endothelium, and macrophages in the transparent chamber of the rabbit's ear. *Anat. Rec.* 50:355.

40. Silver, M. D., and W. E. Stehbens. 1965. The behaviour of platelets in vivo. *Q. J. Exp. Physiol. Cogn. Med. Sci.* 50:241.

41. David-Ferreira, J. F. 1964. The blood platelet: electron microscopic studies. *Int. Rev. Cytol.* 17:99.

42. Rand, M., and G. Reid. 1951. Source of serotonin in serum. *Nature (Lond.)*. 168:385.

43. Tranzer, J. P., M. DaPrada, and A. Pletscher. 1966. Ultrastructural localization of 5-hydroxytryptamine in blood platelets. *Nature (Lond.)* 212:1574.

44. Baker, R. V., H. Balaschko, and G. V. R. Born. 1959. The isolation from blood platelets of particles containing 5-hydroxytryptamine and adenosine triphosphate. *J. Physiol. (Lond.)*. 149:55.

45. Holmsen, H. 1965. Collagen-induced release of adenosine diphosphate from blood platelets incubated with radioactive phosphate in vitro. *Scand. J. Clin. Lab. Invest.* 17:239.

46. Grette, K. 1962. Studies on the mechanism of thrombin-catalysed hemostatic reactions in blood platelets. *Acta. Physiol. Scand. Suppl.* 56:195.

47. Niewiarowski, S., and D. P. Thomas. 1970. Platelet release reaction: the effects on the vessel wall. *In Vascular Factors and Thrombosis.* E. Keller, K. M. Brinkhous, R. Biggs, N. F. Rodman, and S. Hinnom, editors. F. K. Schattauer-Verlag, Stuttgart, W. Germany. 199.

48. Weisbach, H., and B. G. Redfield. 1960. Studies on the uptake of serotonin by platelets. *In Blood Platelets.* Little, Brown and Company, Boston. 393.
49. Zucker, M. B., and J. Borelli. 1959. Platelets as a source of serum acid nitrophenyl phosphatase. J. Clin. Invest. 38:148.
50. Robert, B. Y., G. L. Legrand, G. Dignaud, J. Caen, and L. Robert. 1969. Activité elastolitolytique associée aux plaquettes sanguines. Pathol. Biol. 17:615.
51. Mustard, J. F., H. Z. Movat, D. R. L. MacMorine, and A. Senyi. 1965. Release of permeability factors from the blood platelet. Proc. Soc. Exp. Biol. Med. 119:988.
52. Mustard, J. F., L. Jorgensen, and M. A. Packham. 1970. Formed elements as a source of vascular injury. In Vascular Factors and Thrombosis. F. Koller, K. M. Brinkhous, R. Biggs, N. F. Rodman, and S. Hinnom, editors. Springer-Verlag New York, Inc., New York. 137.
53. Florey, L. 1970. General Pathology. Lloyd-Luke (Medical Books) Ltd., London. 4th edition.
54. Aikawa, M., M. J. Schoenbechler, J. F. Barbaro, and E. H. Sadun. 1971. Interaction of rabbit platelets and leukocytes in the release of histamine. Am. J. Pathol. 63:85.
55. Henson, P. M. 1970. Release of vasoactive amines from rabbit platelets induced by sensitised mononuclear leukocytes and antigen. J. Exp. Med. 131:287.
56. Bak, I. J., R. Hassler, B. May, and E. Westermann. 1967. Morphological and biochemical studies on the storage of serotonin and histamine in blood platelets of the rabbit. Life Sci. Part II Biochem. Gen. Mol. Biol. 6:1133.
57. Holmsen, H., H. J. Day, and H. Stormorken. 1969. The blood platelet release reaction. Scand. J. Haematol. Suppl. 8:13.
58. Miescher, P., and N. Cooper. 1960. The fixation of soluble antigen-antibody complexes upon thrombocytes. Vox Sang. 5:138.
59. Humphrey, J. H., and R. Jaques. 1955. The release of histamine and 5 hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions (in vitro). J. Physiol. (Lond.). 128:9.
60. Henson, P. M. 1969. Immunological induction of increased vascular permeability. II. Two mechanisms of histamine release from rabbit platelets involving complement. J. Exp. Med. 129:167.
61. Henson, P. M. 1970. Release of vasoactive amines from rabbit platelets induced by anti platelet antibody in the presence and absence of complement. J. Immunol. 104:924.
62. Schoenbechler, M. J., and E. H. Sadun. 1968. In vitro histamine release from blood cellular elements of rabbits infected with Schistosoma mansoni. Proc. Soc. Exp. Biol. Med. 127:601.
63. Henson, P. M., and C. G. Cochrane. 1969. Antigen antibody complexes, platelets and increased vascular permeability. In Cellular and Humoral Mechanisms of Anaphylaxis and Allergy. H. Z. Movat, editor. S. Karger AG., Basel, Switzerland. 129.
64. Barbaro, J., and M. J. Schoenbechler. 1970. The nature of the reaction of antigen with lymphocytes from rabbits infected with Schistosoma mansoni on the release of histamine from rabbit platelets. J. Immunol. 104:1124.
65. Siragagian, R. P., and B. Oliveira. 1968. The allergic response of rabbit platelets and leukocytes. Fed. Proc. 27:315.
66. Siragagian, R. P., and A. G. Osler. 1969. Histamine release from sensitised rabbit leukocytes and associated platelet involvement. *J. Allergy.* 43:167.

67. Buckley, I. K., and G. B. Ryan. 1969. Increased vascular permeability. The effect of histamine and serotonin on rat mesenteric blood vessels *in vivo.* *Am. J. Pathol.* 55:329.

68. Majno, G., S. M. Shea, and M. Leventhal. 1969. Endothelial contraction induced by histamine type mediators. *J. Cell Biol.* 42:647.

69. Majno, G., and G. E. Palade. 1961. Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability. An electron microscopic study. *J. Biophys. Biochem. Cytol.* 11:571.

70. Gabbiani, G., M. C. Badonnel, and G. Majno. Intra-arterial injections of histamine serotonin or bradykinin. A topographic study of vascular leakage. *Proc. Soc. Exp. Biol. Med.* 135:447.

71. Austen, K. F., and K. J. Block. 1964. Differentiation *in vitro* of antigen-induced histamine release from complement dependent immune injury. *Complement Ciba Found.* Symp. 281.

72. Majno, G., G. E. Palade, and G. I. Schoeff. 1961. Studies on inflammation. II. The site of action of histamine and serotonin along the vascular tree: a topographic study. *J. Biophys. Biochem. Cytol.* 11:607.

73. Cochrane, C. G., and D. Hawkins. 1968. Studies on circulating immune complexes. III. Factors governing the ability of circulating complexes to localise in blood vessels. *J. Exp. Med.* 127:137.

74. Cochrane, C. G. 1963. Studies on the localization of circulating antigen-antibody complexes and other macromolecules in vessels. I. Structural studies. *J. Exp. Med.* 118:489.

75. Movat, H. Z. 1966. The vascular changes in acute normergic (non-allergic) and allergic inflammation. In Methods and Achievements in Experimental Pathology. 1:245.

76. Knicker, W. T., and C. G. Cochrane. 1968. The localization of circulating immune complexes in experimental serum sickness. The role of vasoactive amines and hydrodynamic forces. *J. Exp. Med.* 127:119.

77. Movat, H. Z., T. Uruihara, M. S. Taichman, H. C. Rowsell, and J. F. Mustard. 1968. The role of injury, inflammation and hypersensitivity. *Immunology.* 14:637.

78. Movat, H. Z., K. Udaka, and Y. Takeuchi. 1970. Polymorphonuclear leukocyte lysosomes and vascular injury. In *Vascular Factors and Thrombosis.* K. M. Brinkhous, F. Koller, R. Biggs, N. F. Rodman, and S. Hinnom, editors. F. K. Schattauer-Verlag, Stuttgart, W. Germany. 211.

79. Horowitz, R. E., L. Burrows, F. Paronetto, D. Drieling, and A. E. Kark. 1965. Immunological observations on homografts. II. The canine kidney. *Transplantation.* 3:318.

80. Hughes, A., and R. S. Tonks. 1962. Intravascular platelet clumping in rabbits. *J. Pathol. Bacteriol.* 84:379.

81. Mustard, J. F., G. Evans, M. A. Packham, and E. E. Nishizawa. The platelet in intravascular immunological reactions. 1st Symposium on Cellular and Humoral Mechanisms in Anaphylaxis and Allergy. S. Karger AG., Basel, Switzerland. 151.
82. Packham, M. A., E. E. Nishizawa, and J. E. Mustard. 1968. Response of platelets to tissue injury. Biochem. Pharmacol. 17:171. (Suppl.)
83. Germuth, F. G. 1953. A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type. J. Exp. Med. 97:257.
84. Dixon, F. J., J. J. Vazquez, W. O. Weigle, L. G. Cochrane. 1968. Pathogenesis of serum sickness. Arch. Pathol. 65:18.