PART IX

TRANSPLANTATION: Graft Rejection
Recipient Monitoring

Chairmen: M Legrain
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IMMUNOLOGICAL DIAGNOSIS OF REJECTION IN HUMAN RENAL ALLOTRANSPLANTED PATIENTS – A PROSPECTIVE STUDY

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Summary

The object of this study has been to evaluate the recipient’s immunological reactivity towards donor lymphocytes in relation to rejection episodes. All recipients (20) of local necrokidneys during 1976 were immunologically monitored immediately before transplantation and subsequently twice weekly for donor-specific complement dependent lymphocytotoxic (CDC) antibodies, antibody dependent cell-mediated cytotoxicity (ADCC) and cell-mediated lympholysis (CML). Experiments were performed until graft removal or dismissal (approx. 1100 patient days). Clinical diagnosis of rejection was made independently of immunological results. All clinically suspected rejection episodes, except one, were checked by microscopy. A positive CML-test accompanied 9 out of 11 rejection episodes; the test was negative on all other occasions. Positive CDC and ADCC tests exhibited no obvious correlation with rejection episodes: positive ADCC may be more frequent in clinically uncomplicated phases. Positive CML did not generally precede clinical graft rejection. Positive CML before transplantation was observed in two cases and was followed by irreversible, accelerated acute rejections. The CML-test may prove a reliable tool in rejection diagnosis and may yield results comparable with graft biopsy without inflicting any risk on the patient.

Introduction

The immunological basis for the rejection of allogenic transplants of organs and tissues has been generally accepted for the last thirty years1. Evidence came then from the observation that a second skin graft placed on a recipient animal would be rejected more rapidly if the recipient had already rejected a first graft from the same donor. As it was further shown that specifically cytotoxic T-lymphocytes occur early after alloimmunisation and further that ‘transplantation-immunity’ could be adoptively transferred to non-transplanted animals by lymphocytes from earlier transplanted animals, and not by serum2,3
it was deduced that allograft rejection was due to alloaggressive lymphocytes.

These experiments were, however, performed by skin-grafting, and it has later been shown unequivocally that humoral antibodies have a decisive role in the rejection of primarily vascularised grafts and that this immunity may be transferred by serum. The impact of humoral antibodies is dramatically underlined by the finding that the presence in the recipient of preformed lymphocytotoxic antibodies active against tissue donor leads to hyperacute rejection of allografts. There is moreover evidence that antibodies play a major role in late rejection, and a correlation has been found between the occurrence of donor specific antibodies and obliterator vascular lesions, and the fact that it has been possible to elute donor specific antibodies from rejected kidneys.

Further studies have, however, revealed that allograft rejection is due to a complex series of events which follow sensitisation of the recipient cell surface components (transplantation antigens) of the donor. Sensitisation may lead to activation of numerous biological systems of effector mechanisms which collectively or separately result in the destruction of the allograft. These may involve lymphocyte activation, coagulation, and destruction of parenchyma by granulocytes, macrophages etc.

In accordance with this, immunological monitoring of the transplant recipient should as a minimum involve techniques detecting both antibody-and cell-mediated immunity as well as techniques on antibody-dependent cell-mediated immunity. By monitoring prior to transplantation (i.e. cross-matching) it would be of importance to be able to disclose immunological memory functions as well as existing effector capacity due to presensitisation by, for example, blood transfusion, pregnancy or previous transplantation. Up till now techniques monitoring immunological memory functions have not been available, other than the technique for lymphocyte-mediated immunity claimed by Stiller et al. to detect memory cytotoxic T-cells (cf. below).

The objectives of this study have been (i) to describe selected immunological patterns of anti-donor reactivity found in renal allotransplanted patients (ii) to evaluate immunological monitoring of allotransplanted recipients in relation to the clinical course in an attempt to provide a more rational basis for the clinical treatment of the transplant patient and (iii) to ascertain whether clinically manifest graft rejection episodes can be predicted immunologically.

Material

The present material comprises 20 different, consecutive renal graft recipients. Transplantation was performed during 1976 in Aarhus using local kidneys from 19 different cadaver donors. Best donor/recipient matches for HLA-A and B antigens were: 4 identical matches, 7 and 9 one and two antigen(s) mismatches, respectively; worst donor/recipient matches were 7, 11 and 2 with one, two and three antigen(s) mismatches, respectively. There were 9 female and 11 male recipients.
Methods

The immune response was followed by three different techniques:

**Complement Dependent Cytotoxicity (CDC) Technique**  This test detects conventional lymphocytotoxic antibodies, presumably directing their activity against the HLA-A,B,C antigens, and possibly also against the ‘Ia-like’ antigens of the B-lymphocytes. The test was performed according to the Kissmeyer-Nielsen and Kjerbye lymphocytotoxic microtechnique\(^{14}\) including incubation for 30 minutes as well as for 60 minutes.

**Antibody Dependent Cell-mediated Cytotoxicity (ADCC) Technique**  This test detects complement independent antibodies exerting their lymphocytolytic capacity through cooperation with unprimed third party lymphocytes generally believed to be Fc-receptor bearing non B and non T lymphocytes, i.e. ‘O’- or ‘K’-lymphocytes\(^{15}\). The test is a \(^{51}\)Cr release assay including incubation for 6 hours. Opinions still differ as to the immunogenetic specificity of ADCC-antibodies. The ADCC test may be a very sensitive technique for detection of anti-HLA-A,B, and C antibodies as normally disclosed by the CDC-technique or may concern the detection of antibodies with yet unknown specificity\(^{16}\).

**Cell-mediated Lympholysis (CML) Technique**  This test detects complement independent, activated T-lymphocytes (T-killer cells). The test is performed as a \(^{51}\)Cr release assay with incubation for 6 hours; details of the technique have been published elsewhere\(^{17}\). Activated T-lymphocytes seem to exert their cytolyis partly via the HLA-antigens and partly via other as yet poorly defined antigenic systems, possibly being organ specific\(^{18}\).

Protocol

Each recipient was immunologically monitored by the techniques mentioned above immediately before transplantation and twice weekly (Mondays and Thursdays) until graft removal or discharge from hospital. In this way 20 consecutive recipients were monitored over a period of approximately 1100 patient days and 11 rejection episodes.

**Donor target lymphocytes** were obtained from spleens of cadaver donors at nephrectomy. The cut spleens were kept under sterile conditions at \(4^\circ\)C in Heps-buffered tissue-culture medium (TC-199 \(\sim\) 200 ml per spleen) until isolation of mononuclear cells could be performed. Isolation was performed directly on the tissue culture medium by Isopaque-Ficoll\(^{19}\). Successful isolation could in this way be performed up to 72 hours after splenectomy yielding approximately \(10^8\) mononuclear cells per spleen with a viability above 90% (dye-exclusion). The yield of cells could be increased to approximately \(10^9\) by gentle homogenisation of the spleen slices. After isolation donor spleen cells were frozen (10% DMSO 50% serum, 40% RPMI) on liquid nitrogen until required for assays. For the CDC assay cells were thawed immediately before use, whereas cells to be used in the CML and ADCC assays were thawed three days before the actual
tests, phytohaemagglutinin (PHA) stimulated and incubated (5% CO₂, 37°C, humidified atmosphere).

Panel target lymphocytes (ie lymphocytes from unrelated individuals highly selected in order to disclose the specificity of antibody or cytotoxic T-lymphocytes) were for the CDC assay obtained from 14–27 individuals while the CML and ADCC assays only included 5 individuals. For all assays selection was primarily based on the HLA-A,B, and C antigens.

Recipient serum and lymphocytes were obtained from venous blood immediately before transplantation and subsequently twice weekly as indicated above.

The CML test was performed on the days of bleeding against donor-target and panel-target lymphocytes, without in vitro sensitisation.

The CDC and ADCC tests were performed jointly after discharge from hospital or graft removal against donor-target and panel-target lymphocytes. ADCC included ‘effector’ lymphocytes from three random, healthy individuals.

ADCC and CML controls involved lymphocytes and sera from 182 random, healthy blood donors tested in parallel for ADCC and CML reactivity. The mean ⁵¹Cr release of these individuals was 1.5% (upper 1% confidence limit: 8.9%).

Diagnosis of rejection was made independently of immunological results. The clinical criteria for rejection were: (1) increased blood creatinine; (2) decreased Na⁺ elimination and urinary output; (3) increased weight; (4) proteinuria; (5) fever; (6) hypertension; (7) enlargement of graft and eventually (8) renography.

All clinically suspected rejection episodes (except one) were either verified or disproved by microscopy. The 11 graft rejection episodes encountered in the 20 patients studied were all classified as acute rejections.

Results

Pre-Transplantation Monitoring

No donor-specific CDC or ADCC antibodies were identified prior to transplantation. As regards CDC antibodies this is of course a condition sine qua non for transplantation. Two recipients were, however, identified who had a positive direct CML against donor target cells. In both cases the transplantation was unsuccessful and the kidneys suffered acute, irreversible rejection within 14 days after transplantation. Both these recipients had been transplanted previously and had received multiple blood transfusions and may well have been immunised as a consequence.

Post-transplantation Monitoring

Five different immunological patterns were observed: (1) negative CDC, ADCC
and CML (7 patients); (2) negative CDC and CML, positive ADCC (4 patients); (3) negative CDC and ADCC, positive CML (4 patients); (4) negative CDC, positive ADCC and CML (1 patient); (5) positive CDC, ADCC and CML (4 patients). All patients falling into immunological group (1) exhibited clinically uncomplicated courses with no graft rejection episodes within the period studied, while patients of groups (3, 4 and 5) all had complicated clinical courses involving graft rejection episodes. Group (2) has no clear clinical relationship. The correlation of the single tests with graft rejection appears in Table I. It is seen that a

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<th>Rejection</th>
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<td>(1) Cell-mediated lymphocytotoxicity (CML)</td>
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<td>(2) Antibody dependent cell-mediated cytotoxicity (ADCC)</td>
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<td>(3) Complement dependent antibody (CDC)</td>
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CML test positive against donor target lymphocytes is highly correlated with graft rejection (p < 0.0001). A specifically positive CML-test accompanied 9 out of 11 clinical rejection episodes and furthermore CML was not positive in patients with no clinical signs of rejection. The donor/recipient HLA-A and B match-grading were not correlated with CML positivity. The patterns of CDC and ADCC reactivity did not in any obvious way correlate with graft rejection.

**Discussion**

Clinical treatment and prophylaxis of rejection episodes is hampered by incomplete knowledge of the many different immunopathological pathways leading to rejection. There is thus a well-defined and urgent need for immunological methods which may be applied easily to the transplant recipient and which permit an analytical clinical judgement.

The data presented in this study underline that the application of three simple immunological techniques encompassing serological as well as cellular immunity produces a pattern of immunological reactivity in the transplant patient that seems to parallel the clinical course in a consistent way. Most promising is the finding that a positive CML-test — indicating the presence in
the recipient of peripherally circulating cytotoxic T-lymphocytes active against
donor antigens — is highly correlated with graft rejection, as 9 out of 11 in vivo
rejection episodes were paralleled by an in vitro positive CML test. This finding
is in concordance with the findings of others\textsuperscript{13,19}.

A general problem with techniques utilising peripheral blood is that the immu-
nologically active agent (antibody or cytotoxic cells) is adsorbed by the target
organ, in this case the renal transplant, leading to false negative reactions\textsuperscript{10,20}.
Since the actual immunity is, however, raised in peripheral lymphatic tissues and
reaches the graft via the blood vessels, this calls for the development of very sen-
sitive immunological methods and optimal times of blood sampling. Further
studies are needed in this respect.

Another complicating factor for the interpretation of the laboratory results
obtained post transplantation may be the immunosuppressive treatment of the
recipient initiated on an empirical, clinical basis. Very little is known about the
exact mechanisms by which these drugs act, and it may be hazardous to apply
the experience obtained in animals to the human situation.

As regards the immunological investigations performed prior to transplanta-
tion, ie humoral and cellular crossmatching, these must in principle involve
techniques detecting the immediate effector-capacity as well as the effector-
potential, especially concentrating on immunological memory-functions. The
techniques described here may fulfil the first objective, while techniques detect-
ing immunological memory have not been unequivocally demonstrated. By
means of an 18-hour CML technique, Stiller et al\textsuperscript{13} claim to be able to delineate
periods — both before and after transplantation — when the recipient is more
responsive immunologically and to predict with confidence the occurrence of
graft rejection episodes. It is further argued by these authors than an 18 hour
CML-technique may detect peripherally circulating memory T-lymphocytes
that may in a matter of hours convert to cytotoxic T-lymphocytes. Our findings
do not confirm these results. First, 18 hours of CML-incubation yield unreliable
results due to the amount of spontaneous chromium release by the target lym-
phocytes. Second, a specifically positive CML-test does not in general precede
clinical graft rejection, but tends to present itself at the same time or following
the acute clinical phase of graft rejection. A positive CML test obtained prior
to transplantation was, however, in the two cases observed, followed by irre-
versible acute graft rejection.

We conclude, that a positive CML test may confirm a clinically suspected
graft rejection and further that the CML-technique may prove a valuable tool
for the distinction between immunological graft rejection and graft malfunction
due to other (eg surgical) causes. In this respect the CML test may yield infor-
mation comparable to graft biopsy without any risk to the patient.

Acknowledgment

This work was supported by the Danish Medical Research Council.
References

1. Medawar, PB (1945) J. Anat. (Lond), 78, 176
4. Clark, DS, Foker, JE, Good, RA and Varco, RL (1968) Lancet, i, 8

Open Discussion

HÄYRY (Helsinki) Did you verify that your killer cells in the blood were T lymphocytes?

HANSEN Yes.

HÄYRY Secondly, to simplify this test, have you by any chance established a continuous cell line from the donor, let us say by using LPS as mitogen, so that you would have target cells always available? You could probably have them always under chromium, and you could simply just wash the target cells and add the effector lymphocytes. In this way you could probably carry out this test more frequently.

HANSEN Yes, we have done so, and we are continuing the test at the present time in these patients, but this study includes only the first period after transplantation.

HÄYRY I am slightly astonished that you cannot see the killer cells in the blood before rejection. The reason is that Peter Roberts, working in my laboratory, has demonstrated that in secondary allograft rejection in mice there is a biphasic profile of killer cells in the blood. The first peak takes place right before the killer cells invade the graft and the second peak when the killer cells disappear from the graft. Would you think that if you monitored these patients somewhat more frequently, you could possibly find killer cells in the blood before rejection?
HANSEN That is a very difficult question to answer because you know in two patients — the patients who had previously been transplanted — it was possible to find these cells before transplantation, but in none of the other patients was this possible and if you look at the patients’ records you will find that this study was divided into two parts. I am the clinician and the tissue-typing laboratory is dealing with the rest of the study, and we did not interfere. So I worked out the clinical schedule beforehand and kept away until the test was done, and then we compiled the study. If you look at that study you will find that the CML positivity is found generally one to two days after you have found clinical signs of rejection and have started your immunosuppressive therapy. I can only say that we could not generally find a positive direct CML before clinical rejection had been evident for one or two days.

LEGRAIN (Paris) Could you comment a little about the practical implications of such tests and about the false positive problems?

HANSEN From the practical point of view this test is not yet suitable for general use because it requires a lot of labour and you have to ensure, by using controls, that you do not get many false positive tests.

JIRKA (Prague) First, how do you verify the rejection episode by histology and the second, do you think that acute rejection and a rejection episode are the same entities?

HANSEN We have performed renal biopsy in all these patients and in this way verified that the rejection had been present in the patient together with clinical signs of rejection: when we state that rejection crisis is present it is done at the ordinary clinic.

PAPADIMITRIOU (Thessaloniki) I think in clinical practice, these tests could be very useful during the stage of prolonged oliguria after transplantation while you have acute tubular necrosis. Did you have any such cases in your study?

HANSEN We had only one case where the diagnosis of rejection was very doubtful. But this patient was treated with Methylprednisolone alone because of fever and tenderness around the graft. You must realise that the donors were local kidney donors and nearly all of the recipients had immediate graft function.