

# Development of immunological assays to monitor pulmonary allograft rejection

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## Abstract

**Background** – At present the diagnosis of pulmonary allograft rejection is made after examination of transbronchial biopsy specimens; this method is highly invasive. A study was performed to determine whether immunological parameters measured in peripheral blood or bronchoalveolar lavage samples correlate with the histological diagnosis of rejection.

**Methods** – Left unilateral pulmonary allotransplantation was performed between dogs. The animals were immunosuppressed with cyclosporin A after transplantation but the dose of this drug was gradually reduced to allow controlled rejection to take place. Rejection was diagnosed histologically. Four immunological parameters were investigated: measurement of lavage derived T cell proliferation in response to limited culture with interleukin 2; measurement of changes in the frequency of donor reactive cytotoxic T lymphocytes; assay of the level of donor cell binding IgG antibody in recipient plasma; and measurement of the antibody dependent cell mediated cytotoxic response to donor cells after labelling with recipient plasma.

**Results** – Assays based on measurement of the function of T cells produced significant results at a time later than the histological diagnosis of severe rejection. The level of donor reactive IgG antibody increased at a time that corresponded closely with the diagnosis of severe rejection. This IgG did not activate the antibody dependent cell mediated cytotoxic effector mechanism to a significant extent.

**Conclusions** – Measurement of parameters of donor specific immunoreactivity can yield data which are indicative of severe pulmonary allograft rejection. These methods make use of samples which can be obtained by minimally invasive methods. Measurement of the plasma level of donor reactive IgG antibody appears to be the most useful assay. However, each of the *in vitro* assays used during this series of experiments was less sensitive to the onset of rejection than was routine histological examination.

or combined heart-lung transplantation. Advances in surgical techniques have resulted in actuarial survival rates of 78% at one year and 65% at two years.<sup>1</sup> Allograft rejection, however, presents an obstacle to long term survival and the processes of pulmonary rejection remain poorly understood.

In combined heart-lung transplantation the heart shows few signs of disease in the absence of ongoing pulmonary rejection.<sup>2,3</sup> Pulmonary tissue contains highly immunogenic bronchus associated lymphoid tissues that are thought to contribute to the enhanced rejection of this organ. Studies of dendritic cells in rat lung have shown that these cells are both numerous and dynamic in their response to local inflammation.<sup>4</sup>

The “gold standard” for pulmonary allograft rejection is the observation of peribronchial and perivascular infiltrates in transbronchial biopsies combined with a failure to detect infectious agents in bronchoalveolar lavage fluid.<sup>5,6</sup> However, biopsy sampling is associated with a risk of morbidity and mortality. Other clinical indicators include radiological changes, pyrexia, reduced gas exchange, and spirometric measurements.<sup>7</sup>

The technique of lavage provides a method for sampling intra-graft immune cells. Canine models of unilateral lung transplantation have shown a correlation between the presence of both non-specific lymphokine activated killer cell activity and specific cytotoxic T lymphocytes in lavage fluid and graft rejection.<sup>8,9</sup> These immune effector cells cannot be isolated from peripheral blood. Donor reactive lymphocytes have been propagated from both biopsy and lavage specimens obtained from lung transplant recipients.<sup>10,11</sup> The reactivity of lavage derived lymphocytes is closely associated with that of cells obtained from biopsy specimens, suggesting that lavage lymphocytes can monitor processes which occur within pulmonary tissue.

The aim of this study was to determine whether immunological parameters measured *in vitro* are indicative of graft rejection in a canine model of unilateral lung transplantation. Donor responsiveness in intra-graft and peripheral components of the recipient immune system was investigated whilst pulmonary rejection was induced in a controlled manner by decreasing the dose of cyclosporin A.

## Methods

### TRANSPLANT SURGERY

Left lung allotransplantation was performed between size matched beagles as previously

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Several end stage pulmonary disorders can be efficiently treated by single lung, double lung,

described.<sup>12,13</sup> Histoincompatibility between donor/recipient pairs was maximised before transplantation by demonstration of positive mixed leucocyte reactivity. Four transplant recipients (numbered 1–4) were studied for 13–16 weeks. A further three transplant operations resulted in technical failure.

Induction immunosuppression was started after surgery with 4 mg/kg intramuscular cyclosporin A (Sandimmun; Sandoz Ltd, Basle, Switzerland) and three intravenous doses of methylprednisolone (10 mg/kg) over the following 24 hours. Maintenance immunosuppression with cyclosporin A commenced once enteral feeding was established (table 1). In addition, recipient animals were given 20 mg frusemide daily to improve graft patency, and 75 mg aspirin on alternate days to counteract side effects of cyclosporin A. Animal 4 received flucloxacillin for the first seven days after transplantation to clear a wound infection; no further antibiotic treatment was necessary.

#### PRESERVATION OF DONOR TISSUE

The non-transplanted donor lung was dissected from the tissue block and immersed in ice cold RPMI 1640 medium (Northumbria Biologicals, Cramlington, Northumberland, UK). Within four hours a 2 cm<sup>3</sup> portion of the tissue was chopped and digested overnight by stirring with type XI collagenase (C9407; Sigma, Dorset, UK) at 1 mg/ml (w/v) in RPMI 1640. After digestion the material was propagated at 37°C in a humid atmosphere containing 5% carbon dioxide in culture medium consisting of RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal calf serum, 1 × 10<sup>-4</sup> mol/l HEPES, 10<sup>5</sup> U/l penicillin (Sigma), and 100 mg/l streptomycin (Sigma). The cultures were split as necessary after detachment by treatment with trypsin-EDTA (Northumbria Biologicals). The cells were characterised morphologically as a mixture of epithelial cells and fibroblasts.

Canine splenic cells were prepared by gently teasing apart spleen tissue. The resultant cell suspension was washed by centrifugation in RPMI 1640. Mononuclear splenic cells were purified by centrifugation (400 g for 30 minutes) over a Ficoll-metrizoate (Lymphoprep; Nycomed, Birmingham, UK) density gradient; the interfacial cells were recovered, washed, and cryopreserved in liquid nitrogen.

#### POST-TRANSPLANTATION SAMPLES

Venous samples of blood were collected at weekly intervals after transplantation into sterile universals containing 100 U preserva-

tive free heparin (Sigma). Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation as described above. Plasma (diluted 1:1 in RPMI 1640) was collected from above the mononuclear cell layer after PBMC preparation and stored at -20°C.

Bronchoalveolar fluid was collected at 14 day intervals after transplantation. Animals were anaesthetised, maintained with propofol, and intubated with a 10 mm cuffed endotracheal tube (Portex, UK). Adequate gas exchange was maintained with jet ventilation and a flexible bronchoscope (Olympus BF-Type 3; Keymed, UK) was introduced and wedged into a limiting bronchus of the lower lobe of the transplanted lung. A lung segment was lavaged with 60 ml RPMI 1640 medium and the cell suspension washed in RPMI 1640, resuspended in culture medium, and the adherent cells (macrophages) removed by incubation on plastic tissue culture flasks (Gibco, Uxbridge, Middlesex) for at least 60 minutes at 37°C in a humid atmosphere containing 5% carbon dioxide.

Following lavage, transbronchial lung biopsy specimens were taken with alligator forceps from the lower lobe and stored in formal saline for routine histological evaluation. The biopsy specimens were graded on a scale from 0 (normal), through 2 (mild; inflammation limited to perivascular and peribronchial infiltration), to 4 (severe; end stage rejection with widespread infiltration and tissue damage). Chest radiography was performed after the biopsy (65 kV, 100 mA, 0.032 seconds) for serial analysis.

All recipient animals were reviewed daily and core temperature, weight, and chest auscultation were measured to assess signs of infection or rejection.

#### IMMUNOLOGICAL MONITORING

##### *IL-2 induced lymphocyte proliferation*

The presence of functional interleukin 2 (IL-2) receptors on the surface of lavage derived cells was shown by measuring their proliferative response cells after stimulation with exogenous recombinant human IL-2 (rIL-2; Boehringer Mannheim, Lewes, East Sussex, UK). Triplicate cultures of 1 × 10<sup>5</sup> adherent cell-depleted lavage cells were incubated in round bottomed microtitre plates in culture medium containing rIL-2 at a concentration of 100 U/ml in a total volume of 200 µl. After culture for 24 hours at 37°C, 1 µCi <sup>3</sup>H-thymidine was added to each well. The cultures were harvested (Dynatech Automash 2000) six hours later for liquid scintillation counting (Packard Tri-Carb 4000). Data are presented as stimulation indexes which were calculated by dividing the counts per minute produced by rIL-2 stimulated cells by that produced by non-stimulated cells.

##### *Limiting dilution analysis*

This assay was performed to measure an increase in the frequency of potentially graft

Table 1 Cyclosporin A dose regimen

Weeks after transplantation	Dose (mg/kg/day)
0–3	20
4–5	15
6–7	10
8–9	7.5
10–11	5
12–13	2.5
≥14	0

damaging donor reactive cytotoxic T cells in the PBMC population of recipient animals. An increase in the frequency of these cells is indicative of specific cytotoxic T cell sensitisation and proliferation.

Limiting dilution assays were established with methodology that has been published previously.<sup>14</sup> Briefly, a series of 20 replicate cultures were established containing limiting numbers of recipient PBMC and  $2 \times 10^5$  irradiated (25 Gy) donor splenic cells. Control assays were established using third party splenic cells. The assays were incubated in the presence of 50 U/ml rIL-2 in round bottomed microtitre plates (Nunc) for 12 days at 37°C in a humid atmosphere containing 5% carbon dioxide.

Lymphoblast target cells were prepared from splenic cells by incubation with 10 µg/ml phytohaemagglutinin (Sigma) for three days. The lymphoblasts were labelled with 200 µCi  $\text{Na}_2^{51}\text{CrO}_4$  (Amersham) for 90 minutes at 37°C, and then washed twice in RPMI 1640, once in complete medium, and were adjusted to  $2 \times 10^4$  cells/ml in the complete medium.

Cytotoxic activity in the limiting dilution assay microcultures was detected by incubation of the assay cells for four hours with  $2 \times 10^3$  chromium-51 labelled donor or third party lymphoblasts. Culture supernatants (100 µl) were then harvested for gamma counting (1272 Clinnigamma, Wallac LKB, Milton Keynes, UK; 70 g for five minutes). The cultures were considered positive when they produced a release of  $^{51}\text{Cr}$  which exceeded the mean plus three standard deviations of spontaneous release control cultures which were initially established with no recipient PBMC. The frequency of donor reactive cytotoxic T lymphocytes was calculated by the maximum likelihood method.<sup>15</sup>

#### *Quantification of IgG specific for donor pulmonary cells*

Monolayers of canine pulmonary cells were

detached from the tissue culture flask with trypsin-EDTA, washed by centrifugation, and adjusted to  $2 \times 10^6$  cells/ml in culture medium. Duplicate samples of pulmonary cells were incubated with an equal volume of the plasma sample for 20 minutes at 37°C. After washing by centrifugation in a balanced salt solution (Isoton; Coulter Instruments, Luton, Bedfordshire, UK) the cells were incubated with an optimal dilution of fluorescein isothiocyanate conjugated, affinity purified rabbit anti-dog IgG (Sigma) for 20 minutes at 37°C. The pulmonary cells were then washed again and resuspended in Isoton. The amount of IgG bound to 10 000 donor or third party pulmonary cells was quantified by fluorescence using a FACS 420 microfluorimeter (Becton Dickinson, Cowley, Oxford, UK).

#### *Quantification of antibody dependent cell mediated cytotoxicity*

Pulmonary cells were detached from tissue culture flasks and labelled with 200 µCi  $\text{Na}_2^{51}\text{CrO}_4$  as above. The cells were adjusted to  $4 \times 10^4$  cells/ml and a 50 µl volume was incubated with 50 µl of the appropriate heat inactivated (56°C for 30 minutes to inactivate complement) plasma sample in round bottomed microtitre plates for 30 minutes at 37°C. Then 100 µl of canine PBMC was added to each well and incubation continued for a further four hours, followed by centrifugation at 70 g for five minutes and 100 µl of supernatant was removed for gamma counting (1272 Clinnigamma, LKB). The spontaneous release of  $^{51}\text{Cr}$  from the target cells was determined by adding 50 µl  $^{51}\text{Cr}$ -labelled pulmonary cells to 150 µl of culture medium containing no effector cells; maximal release of  $^{51}\text{Cr}$  was determined after two cycles of freezing ( $-70^\circ\text{C}$ ) and thawing the target cells. The percentage release of  $^{51}\text{Cr}$  was calculated by the equation:

$$\% \text{ specific } ^{51}\text{Cr} \text{ release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

#### STATISTICAL ANALYSES

The results of lavage cell proliferation assays, antibody binding assays, and antibody dependent cell mediated cytotoxicity assays were analysed by the two sample Student's *t* statistic (Minitab Statistical Software; Clecom, Birmingham, UK). The results of limiting dilution assays of cell frequency were compared by maximum likelihood estimation using the GLIM statistical package (Numerical Algorithm Group, Royal Statistical Society, Oxford, UK).

#### Results

Each of the animals developed histological evidence of pulmonary allograft rejection (fig 1) as the dose of cyclosporin A was reduced below 15 mg/kg/day (table 2). The results of chest radiography paralleled the histological findings. In general an early infiltrate

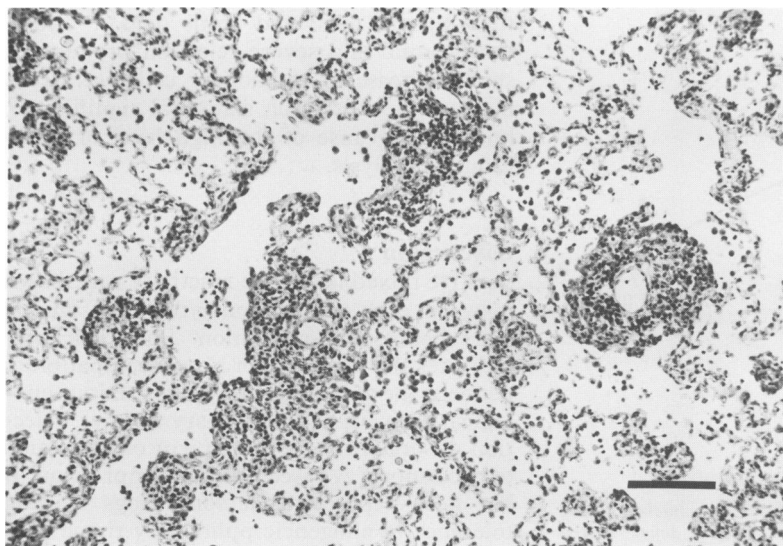


Figure 1 Histological preparation of transbronchial biopsy specimen showing peribronchial and perivascular mononuclear cell infiltration characteristic of pulmonary allograft rejection. Stain: haematoxylin and eosin. Bar = 100 µm.



Table 2 Histological grade of transbronchial biopsy and radiographic findings in recipient animals after transplantation

Weeks	Histological and radiographic findings			
	Dog 1	Dog 2	Dog 3	Dog 4
1	NT	0§	NT	NT
2	0	NT	0	0
3	NT	NT	NT	NT
4	1	NT	0	0
5	0	0	NT	NT
6	NT	NT	0	2*
7	NT	0*	2	NT
8	2*	NT	NT	2†
9	NT	2	NT	NT
10	NT	NT	2*	NT
11	4	4†	NT	4††
12	NT		NT	
13	4†		4	
14			NT	
15			4†	
16			4††	

NT = not tested.

\* First sign of infiltrate on chest radiography.

† Experiment terminated.

‡ Pulmonary consolidation.

§ Histological grading on a scale from 0 to 4.

was observed by histologically defined grade 2 rejection; the lung became consolidated (radio-opaque) by grade 4.<sup>16</sup> Bacteriological analysis of lavage fluid revealed no growth in 69% of samples. Specifically, Gram negative organisms were isolated from 23% of the samples (six of 35 contained *Pseudomonas* and two contained *Enterobacter cloacae*; *Staphylococcus simulans* (Gram positive) was isolated from one sample. Postmortem examination did not reveal significant infection related disease.

The proliferative response of non-adherent lavage cells after 24 hour culture in rIL-2 was determined. In total 28 lavage samples were examined by this assay but the only significant lymphoproliferative responses were observed during severe rejection when doses of cyclosporin A were at their lowest levels. Maximum stimulation indices at this time ranged from multiples of 2.4 (animal 1; week 12;  $p < 0.001$ ), 2.7 (animal 4; week 13;  $p < 0.05$ ), 4.8 (animal 2;

week 11;  $p < 0.001$ ), to 12.3 (animal 3; week 13,  $p < 0.05$ ) of the background incorporation of tritium labelled thymidine by non-stimulated cells. No correlation was observed between the number of cells recovered by lavage of the transplanted lung and the rejection status of that lung (data not shown).

PBMC concentrations after transplantation were regularly assayed to determine the frequency of donor reactive cytotoxic T lymphocytes. Frequencies of  $< 1/100\,000$  cells were regarded as negative. Reactivity was observed in animals 1, 3, and 4 during the latter stages of allograft rejection when the administration of cyclosporin A was minimal. The frequency of donor reactive cytotoxic T lymphocytes in animal 1 became measurable ( $1/72\,500$ ) 89 days after transplantation; end stage rejection was found when the experiment was terminated on day 91. The frequency for animal 4 also became measurable ( $1/6200$ ) during the week before termination of this experiment. Longitudinal frequency data for animal 3 are shown in fig 2; in this recipient the frequency of donor reactive cytotoxic T lymphocytes increased from  $< 1/100\,000$  to  $1/70\,400$  by day 85 after transplantation. This frequency increased significantly ( $p < 0.001$ ) to  $1/16\,100$  on day 96 and  $1/10\,200$  by day 115 after transplantation. This latter value reflects at least a 10 fold increase in the proportion of donor reactive cytotoxic T lymphocytes during the course of pulmonary allograft rejection. Changes were not observed in the frequency of cytotoxic T lymphocytes reactive with third party splenic cells; this is indicative of the specificity of the frequency assay.

Concentrations of IgG specific for donor pulmonary cells consistently increased significantly ( $p < 0.01$ ) from prerejection levels in the plasma of all of the recipient animals during severe allograft rejection (fig 3); there was no increase in the labelling of third party pulmonary cells (data not shown).

The ability of these IgG antibodies to initiate pulmonary cell lysis by an antibody dependent cell mediated cytotoxic mechanism was investigated. The results in table 3 show that plasma recovered from dogs 1 and 3 during mild and severe rejection produced a small increase in pulmonary cell lysis. This increase did not reach statistical significance, however, because of the high level of background cytotoxicity.

## Discussion

Positive mixed leucocyte reactivity was shown between each of the donor/recipient animal pairs before transplantation. Such reactivity indicates the existence of histoincompatibility between the animals and provides an immunological basis for the pulmonary allograft rejection observed for each recipient on reduction of cyclosporin A based immunosuppression. It has been reported that some dogs develop tolerance to allogeneic pulmonary tissue during a limited period of immunosuppression after transplantation.<sup>17</sup> No evidence for such tolerance induction was found.

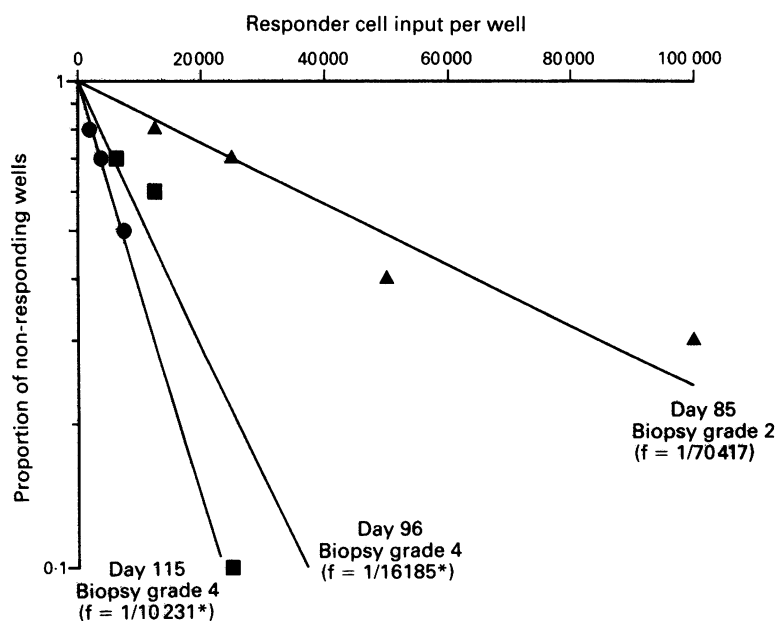


Figure 2 Results of limiting dilution analysis of peripheral blood mononuclear cells (PBMC) taken at three time points from recipient 3. The frequency of donor reactive cytotoxic T lymphocytes in the PBMC at each time point is indicated in parentheses. The frequency values exceeded  $1/100\,000$  cells before day 85 after transplantation. The asterisk indicates that the frequency value deviates significantly ( $p < 0.0001$ ) from the value at day 85.

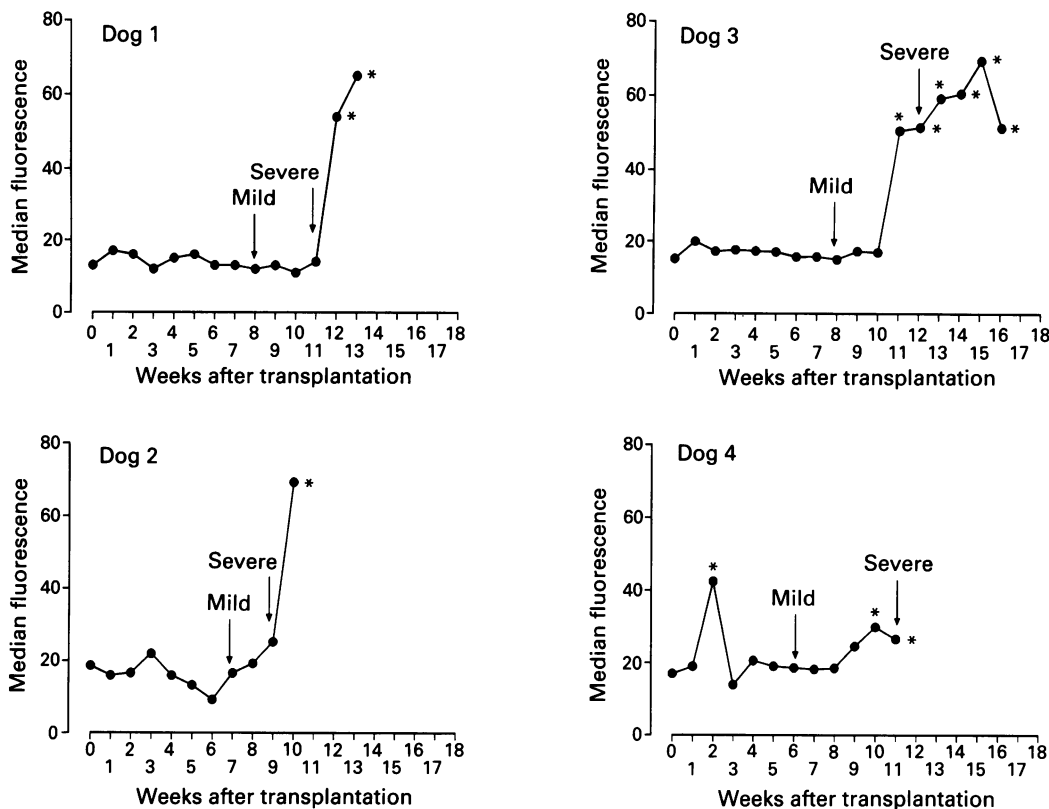


Figure 3 Results of flow microfluorimetric assay of labelling of donor pulmonary cells by IgG in recipient plasma in four dogs. The median fluorescence value is proportional to the amount of bound IgG. Each point represents the mean of triplicate determinations. Arrows indicate the first diagnosis of mild and severe pulmonary allograft rejection. The asterisks indicate values that deviate significantly ( $p < 0.001$ ) from the results obtained before rejection.

A model of non-drug modified acute pulmonary allograft rejection in rats has been used to show that IL-2 induced proliferation of lavage derived cells suggests graft rejection.<sup>18</sup> This assay reflects the number of activated, and therefore IL-2 responsive, T cells within the bronchoalveolar space. Our results showed IL-2 responsiveness within the lavage derived cell population. However, lymphocyte proliferation was only observed consistently when immunosuppression was effectively absent, when the lung showed very severe rejection which was probably irreversible.

The limiting dilution assay has been used extensively to measure the frequency of donor reactive lymphocytes after organ transplantation.<sup>19-21</sup> The technique is both highly specific

and, as it uses peripheral blood samples, minimally invasive. In the present study the initial frequency of donor reactive cells was too low to measure in PBMC from each of the recipients. This may reflect a degree of histocompatibility between semi-inbred dog strains such as the beagle. An increase in the frequency of donor reactive cytotoxic T lymphocytes was observed in three of the recipients late in the rejection process; at this time the disease was well advanced.

All of the recipients developed IgG antibodies in their plasma which were capable of binding to donor cells. The amount of this antibody increased significantly at a time after transplantation that corresponded closely with the onset of grade 4 allograft rejection. Furthermore, this assay is both specific, as the antibody being measured only binds to donor cells, and minimally invasive as it makes use of very small samples of blood plasma.

Donor reactive antibody is generally associated with hyperacute rejection. It is becoming increasingly apparent, however, that newly synthesised antibody may have an active role in the acute and chronic stages of rejection.<sup>22,23</sup> Antibody of the IgG class may mediate graft cell damage by activation of cytotoxic mechanisms. One of these mechanisms is the antibody dependent cell mediated cytotoxicity response involving CD16 positive natural killer cells. This has been implicated in renal allograft rejection.<sup>24,25</sup> The results of the present study, however, show that donor reactive antibodies produce no significant antibody dependent cell mediated cytotoxicity of cultured donor pulmon-

Table 3 Ability of IgG in plasma to activate antibody dependent cell mediated cytotoxicity of cultured donor pulmonary cells. Results are shown for plasma collected preoperatively and during periods of mild and severe pulmonary rejection, expressed as mean (SD) of three samples

Recipient	Time of sample	Specific cytotoxicity of pulmonary cells produced by diluted plasma (%)		
		1:1	1:10	No plasma
Dog 1	Preoperative	29.5(6.5)	13.6(6.6)	13.7(7.2)
	Week 9*	35.6(14.6)	31.3(3.7)	
	Week 13†	36.7(12.6)	15.9(4.2)	
Dog 2	Preoperative	10.9(3.5)	12.5(4.0)	3.4(2.8)
	Week 7*	5.6(5.0)	9.6(7.1)	
	Week 11†	7.3(5.8)	0.8(4.5)	
Dog 3	Preoperative	19.5(7.7)	20.0(2.0)	17.0(1.5)
	Week 7*	30.4(4.7)	25.6(4.6)	
	Week 16†	27.6(1.5)	24.5(5.6)	
Dog 4	Preoperative	14.3(5.0)	12.3(2.7)	10.5(1.2)
	Week 6*	11.9(2.0)	11.8(4.0)	
	Week 10†	13.5(2.1)	8.4(5.7)	

\* Mild pulmonary rejection.  
† Severe pulmonary rejection.

ary cells. This does not exclude the possibility that graft damage may be mediated by the activation of complement.

We have shown that minimally invasive assays of immune function can be used to examine the rejection status of pulmonary allograft tissue. In all cases radiographic examination revealed pulmonary infiltration before rejection could be detected in vitro. Unlike radiographic examination, however, the results of antigen specific immunological assays are unlikely to be perturbed by graft infection. Assays of T lymphocyte function were relatively insensitive, giving significant data only during periods of histologically defined severe graft rejection. Measurement of donor reactive antibody levels yielded data most closely related to the diagnosis of severe rejection by routine histological methods. However, antibody levels did not increase significantly during histologically defined mild rejection. It is clear that in vitro assays must be further improved if they are to take the place of invasive histological measures of pulmonary allograft rejection.

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