ALTERED IMMUNOREGULATORY FUNCTION IN LONG-TERM RENAL ALLOGRAFT RECIPIENTS

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Summary

In order to determine immunoregulatory lymphocyte subsets in patients with long surviving renal allografts, a study utilising functionally characterised monoclonal antibodies with analysis by flow cytometry using a Fluorescence Activated Cell Sorter was carried out. Cells from 35 patients with allograft survival from one to 15 years were analysed with monoclonal antibodies for the following markers: inducer-helper, cytotoxic-suppressor, monocytes, Ia, IgG and IgM. Control groups consisted of long-term dialysis patients and a group of normal individuals. The results show a strikingly significant difference between the long-term allograft recipients and the control population in terms of the inversion of the normal inducer-helper to cytotoxic-suppressor ratio. Additionally, there were significant differences in the number of Ia$^+$ cells in long-term transplant patients and dialysis patients compared with normals. No impressive differences were found in the numbers of Ig bearing cells. Thus the long-term allograft state is a clear example of an overabundance of cellular suppression and may explain many of the general phenomena seen in surviving recipients.

Introduction

Long-term recipients of renal allografts must be maintained on chronic immuno-suppressive medication and have various previously described deficits in immune function [1]. Some of these may be potentially life threatening and pose increased risks which relate to malignancy and susceptibility to infection. We have documented in a large series of patients that the progressive development of anergy to delayed hypersensitivity skin tests is associated with the long term allograft state [2]. As well, in long term patients there is an increased risk of malignancy of the order of 6.5% encompassing a 10 year cross-sectional study from our centre and anergy is a marker for malignancy as well as early and occasional late lethal and non-lethal complications of bacterial sepsis. In spite of the fact that most patients now enjoy long term renal allograft function if allografts are not lost in the first
post-transplant year, there is little doubt that they have an altered immunological state. The purpose of this study was to determine whether or not an alteration in peripheral blood immunoregulatory lymphocyte subsets could be demonstrated in recipients of renal allografts who are long-term post-transplant by making use of the new technique of Fluorescence Activated Cell Sorter analysis using monoclonal antibodies to lymphocyte subsets.

Material and methods

Thirty millilitres of heparinised blood was drawn from 35 recipients of long-term transplant recipients at risk from one to 15 years following renal allotransplantation and from 12 normal blood donors and 19 patients on long-term haemodialysis. Normal control values had previously been established and the procedures followed in this laboratory have been described [3].

Lymphocytes were isolated on a ficoll-hypaque gradient and aliquots were stained for immunofluorescence with appropriate reagents. For this study, the reagents that were used were FITC rabbit anti-human IgG and FITC rabbit anti-human IgM (Cappel Labs., Cochranville, PA); A.TH anti-A.TL mouse alloantisera (Cedarlane Labs., Hornby, Ont.) which crossreacts with human Ia antigens, the monoclonal antibodies (kindly supplied by Mr W Rhodes, Ortho Diagnostics, Raritan, NJ) OKT3 which labels peripheral blood T lymphocytes, OKT4 which labels peripheral blood T inducer-helper cells, OKT8 which labels peripheral blood T cytotoxic-suppressor cells and OKM2 or anti-monocyte antibodies (Bethesda Research Labs, Bethesda, MD) which labels a peripheral blood subset of monocytes. The latter five antibody reagents were mouse IgG, therefore after incubation with cell suspensions and appropriate washing, the cells were incubated with F(ab')2 rabbit anti-mouse IgG. A control mouse protein at appropriate concentration was also used for incubation with cells followed by staining with the F(ab')2 FITC anti-mouse reagent to monitor nonspecific staining. After washing, cells were fixed with 1% paraformaldehyde and 50,000 cells were subsequently analysed by flow cytometry. A FACS III (Becton-Dickinson FACS Systems, Sunnyvale, CA) was utilised in all studies. The principles of analysis using the FACS has previously been described by Loken and Herzenberg [4]. All dot plot displays from the FACS show light scatter values on the X axis and the corresponding fluorescence intensity value for each cell on the Y axis. The histogram data was recorded on cassettes using a Texas Instrument 733 data terminal. The cassettes were subsequently read into a PDP11/50 computer (Digital Equipment Corp., Maynard, MA) where programmes for histogram integration and plotting facilitated computation of the fraction of cells with increased size and increased fluorescence above background. Statistical analysis of the data was done using Student’s t-test.

Results

It can be seen in Table I that the previously studied normals and the long-term dialysis patients showed similar results in the relative ratios of inducer-helper and cytotoxic-suppressor cells with ratios of 2.02 ± 0.27 and 1.83 ± 0.14. In the long-
TABLE I. Lymphocyte profiles before and after transplantation

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>% Ia⁺</th>
<th>% IgM⁺</th>
<th>% IgG⁺</th>
<th>Inducer-helper/Cytotoxic-suppressor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis controls</td>
<td>19</td>
<td>15.8 ± 1.3</td>
<td>7.0 ± 0.9</td>
<td>3.9 ± 0.8</td>
<td>1.83 ± 0.14</td>
</tr>
<tr>
<td>Long-term transplant</td>
<td>35</td>
<td>20.7 ± 4.1*</td>
<td>6.4 ± 0.7</td>
<td>4.2 ± 0.8</td>
<td>1.28 ± 0.10†</td>
</tr>
</tbody>
</table>

Normal values:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Inducer-helper/Cytotoxic-suppressor</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Ia⁺</td>
<td>9.5</td>
<td>± 1.7</td>
<td></td>
<td></td>
<td>2.02 ± 0.27</td>
</tr>
<tr>
<td>% IgM⁺</td>
<td>6.4</td>
<td>± 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IgG⁺</td>
<td>2.3</td>
<td>± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Long-term versus normals: \( t = 2.53, \text{ df } = 44, \text{ p } = 0.015 \)
Long-term versus dialysis controls: \( t = 1.15, \text{ df } = 41, \text{ p } = 0.258 \)
Dialysis controls versus normals: \( t = 2.96, \text{ df } = 24, \text{ p } = 0.007 \)
† Long-term versus normals: \( t = 2.58, \text{ df } = 14, \text{ p } = 0.022 \)
Long-term versus dialysis controls: \( t = 3.23, \text{ df } = 37, \text{ p } = 0.003 \)
Dialysis controls versus normals: \( t = 0.62, \text{ df } = 18, \text{ p } = 0.543 \)

term transplant patients the ratio of inducer-helper to cytotoxic-suppressor cells was 1.28 ± 0.10 which is considerably below that of the normal and long-term dialysis patients. This achieved statistical significance with a p value of 0.022 and 0.003 respectively. In addition, there were differences in the number of Ia cells between normals and the groups of dialysis and long-term patients. These differences were statistically significantly different with p values of 0.007 and 0.015 respectively. Whether these differences between the groups are related to increase in relative numbers of monocytes, B or T lymphocytes is as yet unclear. Differences in IgG and IgM bearing cells were not found to be significant.

Discussion

It is generally believed that functionally distinct immunoregulatory T lymphocyte subsets determine the nature of T-B interactions in the immune response [5, 6]. By use of subset enumeration techniques with functionally characterised monoclonal antibodies [7] our results clearly indicate that there is an alteration in the immunoregulatory subsets in long-term patients following renal allotransplantation. Previous study has indicated that there is no large difference in the total number of T cells enumerated by rosettes or functionally when tested with mitogens in these patients [2]. While total T cell numbers may be somewhat less than normal, the ratio of inducer-helper to cytotoxic-suppressor cells was found to be significantly altered with a relative increase in the number of cytotoxic-suppressor lymphocytes. These observations clearly indicate that the long-term allograft state is modified by a change in the normal immunoregulatory balance and thus suggests that some of the long-term observations on immunodeficiency in these patients could be related to this measurable imbalance. Whether or not the differences in Ia⁺ cells observed in these populations resolves itself into an important discrimi-
natory marker remains to be seen.

It needs to be defined whether there are important in vivo correlations between particular functional responses and the ratios of these T lymphocyte subsets as well as patterns of subsets or absolute numbers in the development of long-term infectious complications or malignancy post-transplant. Whether or not the state of decreased reactivity to delayed hypersensitivity skin test antigens as well as complete anergy which is noted with increasing frequency on an annual basis in our population can be attributed to the increased state of suppression in these patients will be important to ascertain in the future.

We have recently reported lymphocyte subset data on a similar state of immunosuppression in patients immediately following renal transplantation [3]. A clear definition of the various states of rejection, infection, effects of surgery, blood transfusion and other confounding variables can be easily documented by using this technology. While there are a number of aspects that are to be resolved in terms of the amount of raw data generated on a single cell sample by this technology, problems of analysis of cells of patients compared with normals, and problems of indirect immunofluorescence, the latter can be dealt with by using directly conjugated monoclonal antibodies either with FITC or biotin and reacted with FITC-avidin providing improved resolution of subpopulations. It is significant that by using this type of technology, combining analysis using the Fluorescence Activated Cell Sorter with monoclonal antibodies to lymphocyte subsets that a powerful new tool for clinical monitoring has emerged. It should be possible with additional refinements in technique to define more clearly and classify all states of renal transplant recipients.

Acknowledgments

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References

1 Guttmann RD. *N Engl J Med* 1979; 301: 975 and 1038
4 Loken MR, Herzenberg LA. *Ann NY Acad Sci* 1975; 254: 163
5 Cantor H, Gershon RK. *Red Proc* 1979; 38: 2058

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Open Discussion

LESKI (Chairman) Were all the patient in this study treated with cyclophosphamide, because I remember something about long-term effects of cyclophosphamide in patients not treated for transplantation?

GUTTMANN The patients may receive up to 2.4g of cyclophosphamide in the first month. That would be all. These are patients that are now one to 15 years post-transplant and have not received any other cyclophosphamide. The other part of the regimen would be fairly standard. About 10 per cent of our patients have received antilymphocyte or antithymocyte serum for acute rejection episodes, but in general it is a steroid-azathioprine protocol.

LESKI But would you say that patients treated without cyclophosphamide would have the same results?

GUTTMANN Yes, two and a half grams is a negligible amount in terms of the total amount of prednisone and azathioprine which are in decagrams in the first year, and after one year the patients are on 10 or 15mg of prednisone and 100 to 125mg of azathioprine.

TRAEGGER (Lyon) Did you compare your ratio between long-term surviving transplant and short-term surviving transplant?

GUTTMANN Yes, in fact, we have tried to do a number of correlations and they are not of significance. The ratios of helper to suppressor cells in the patients with very long-term or one year, two year survival were in the same range, and with other clinical correlations such as age, time on dialysis and so on we couldn’t find statistically significant correlations by doing regressions.

TRAEGGER When you are using antithymocyte globulin in these patients, how long should you wait before testing your patient? Is it of no value during the treatment?

GUTTMANN Well, this study really concerned the patients after one year, and as might be apparent, what you find will be very dependent on how many T cells there are in the peripheral blood when you make the measurement, and in the acute period, including times when the antithymocyte serum is used, T cell levels are extremely low. But one can still make the measurement even when the levels might be 200 or 300 per ml of blood.