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# The Immunological Significance of Cellular Infiltrates in Chronic Rejection of Human Kidney Transplants

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*Summary.* Fifteen transplanted human kidneys with clinical and histological signs of chronic rejection were examined microscopically for cellular infiltration. Three normal kidneys were used as a reference. All infiltrating cells were classified and counted. The average number of cells per 10 microscopic fields was called the relative density of cellular infiltrates. Differences in the densities of different cell classes and changes in the cellular infiltration of the grafts were regarded as cellular expressions of the immune response.

Summarizing our results, we conclude that:

1) the chronic immunological rejection of transplanted human kidneys is essentially caused by immunocompetent cells;

2) plasma cells develop in the graft itself;

3) the immunocompetent cell population tends to be purely and simply made up of plasma cells;

4) therefore, the true "effector cell" among the immunocompetent cells may be the plasma cell — at least in the chronic rejection of transplanted human kidneys.

Cleaved lymphocytes were the most frequent of the infiltrating cells found in the transplants. However, their role is not yet clear.

Much has been published about immunopathological lesions in transplanted kidneys (Hamburger and Dormant, 1968; Zollinger *et al.*, 1973). However, we know of only one report on studies of the cytokinetics of infiltrates in transplanted kidneys using tritium-labeled thymidine ($H^3T$) (Lindquist *et al.*, 1969). The authors did not classify the different cells which appeared. This encouraged us to differentiate and quantify the infiltrating cells in transplanted human kidneys with signs of chronic rejection and to compare the results to those for normal kidneys. In our opinion, the cellular immune reaction against grafts should be reflected by changes in the amount if these cells and in the relation between the amounts.

## Material and Methods

Fifteen transplanted and 3 normal human kidneys were examined microscopically. The grafts had been surgically removed 90–660 days after transplantation due to symptoms of approaching rejection, chiefly dysfunction. Histologically the transplants showed signs of chronic rejection, such as focal interstitial fibrosis and infiltration of mononuclear and polymorphonuclear cells, often stenosing fibrosis or endothelial swelling of the arteries, and atrophy of the tubules.

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*Histological Methods.* Tissue from both the normal and the transplanted kidneys was fixed in 5% Formalin and embedded in methacrylate. 1.5 μm thick sections were prepared and stained with HCl-Giemsa.

The slides were examined at a magnification of × 1000. 40–240 fields per kidney were looked at. We classified all of the infiltrating cells and then determined the average number of cells per 10 fields. This value was called the relative density of the cellular infiltrates. We considered not only the cells found in the interstitium but also those lying in the lumen of vessels or tubules. The relative density of cellular infiltrates represented our parameter for comparison.

This method yields reliable parameters for comparison, even though there is a relationship between the number of cells determined and the thickness of the slide. If the ratio of the diameter of the cells to the thickness of the slide is large, there is definitely a significant difference between the measured and the true density of cellular infiltrates (Henning, 1963; Treff, 1967). However, the difference in this deviation from cell class to cell class will be comparatively very small.

We therefore evaluated both the total relative density of all of the infiltrating cells and that of each cell class. Altogether we analyzed 2600 fields and classified 27800 cells.

*Statistical Methods.* We compared the densities of all infiltrating cells and of each cell class in the transplanted kidneys with those in the reference kidneys using the Wilcoxon Test (Wilcoxon, Wilcox, 1964). For a graphic demonstration of the differences between the controls and grafts we determined the arithmetic average of the relative densities. The ratios between the cell densities in the transplants and those in the normal kidneys proved to be another informative parameter. We also analyzed the correlations between various cell classes (Sachs, 1968).

## Results

### *A. Relative Density of All Infiltrating Cells*

The relative density of all infiltrating cells was significantly greater in the grafts than in the reference kidneys ($T = 6$; P 0.01). The average value for the normal kidneys amounted to 31.87, whereas that for the transplanted kidneys was 140.33 (Fig. 1).

### *B. Relative Densities of the Different Cell Classes*

The Wilcoxon Test showed that the found differences of the relative densities of the following four cell classes in the controls and grafts were not significant.

1. The average relative densities of *neutrophil polymorphonuclear leukocytes* in the transplanted kidneys (4.38) and in the normal kidneys (4.16) were nearly the same (Fig. 2).

2. The average relative density of *eosinophil polymorphonuclear leukocytes* was greater in the grafts (0.65) than in the reference kidneys (0.11) (Fig. 2).

3. The average relative density of *monocytes* was smaller in the grafts (1.74) than in the normal kidneys (3.67) (Fig. 2).

4. The average relative density of *mast cells* in the grafts (2.75) was greater than in the reference kidneys (0.22) (Fig. 2).

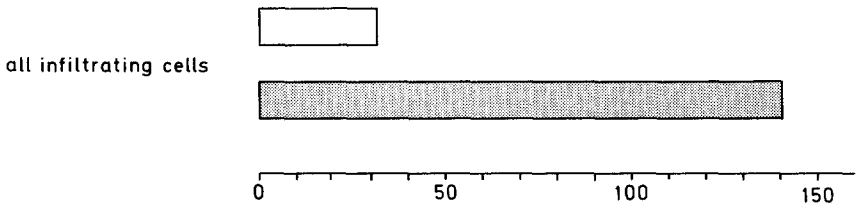


Fig. 1. Arithmetic averages of the relative density of all infiltrating cells. The white oblong denotes the value for the reference kidneys, the gray oblong for the grafts

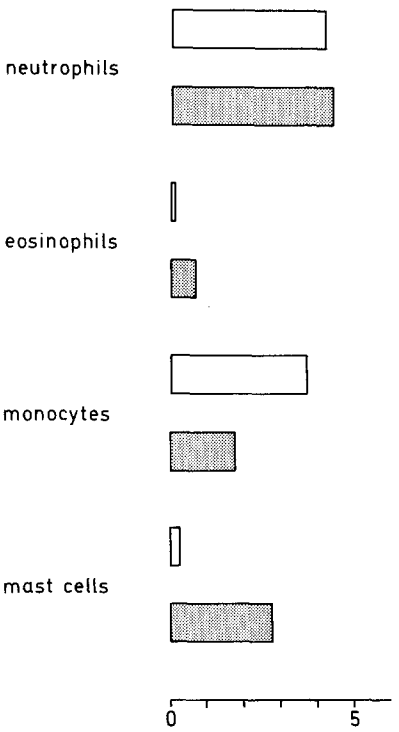


Fig. 2. Arithmetic averages of the relative densities of neutrophils, eosinophils, monocytes, and mast cells. The white oblongs represent the values for the reference kidneys, the gray oblongs for the grafts

Other cells found in the transplanted kidneys included small and large lymphocytes, cleaved lymphocytes, basophil stem cells, plasmablasts, proplasmacytes, and plasma cells. Before presenting the results for these cells, we shall define their most important morphological characteristics, since there is no general agreement on the classification.

*Large lymphocytes* are only slightly different from *small* lymphocytes (Fig. 3, *1* and *2*). In addition to the difference in size of the cells, the nucleus of the large lymphocytes is less compact and usually shows a prominent nucleolus. The most important feature of cleaved lymphocytes (Lukes and Collins, 1973) (Fig. 3, *3*) is

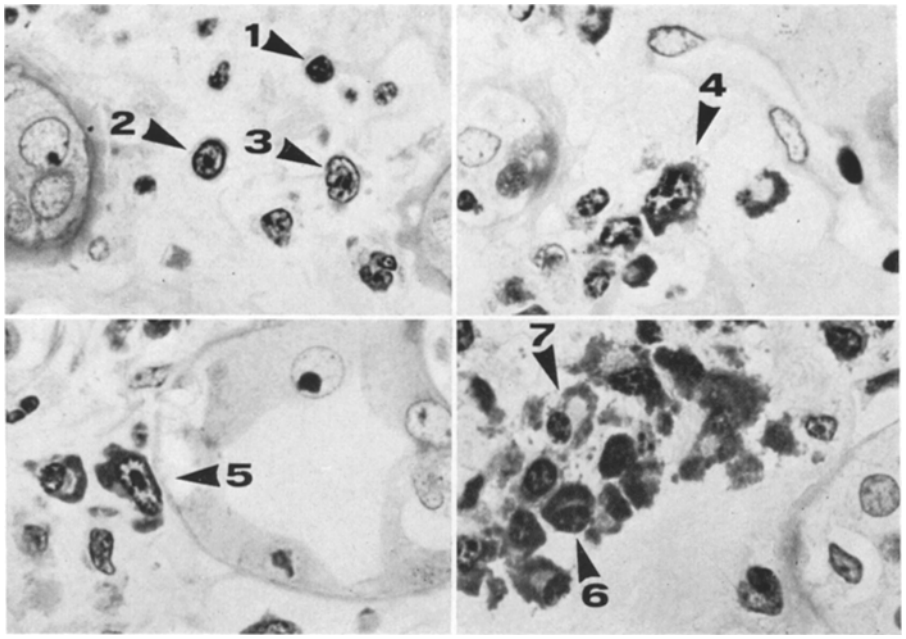


Fig. 3. Immunocompetent cells: 1 small lymphocyte, 2 large lymphocyte, 3 cleaved lymphocyte, 4 basophil stem cell, 5 plasmablast, 6 proplasmacyte, 7 plamsa cell. HC-Giemsa, ×800

the indented nucleus. Therefore they sometimes look like monocytes. However, cleaved lymphocytes are generally smaller and the nuclear chromatin is denser, especially near the nuclear membrane. The cytoplasma is not as wide and is slightly basophilic.

Plasma cells and their precursors were classified as *plasmablasts*, *proplasmacytes*, or *plasma cells* (Lennert, 1961). Instead of differentiating these cells according to the diameter of the nucleus (Sainte-Marie, 1966), we used the following criteria: the nucleocytoplasmic ratio, the size and shape of the nucleus, the chromatin structure, the size of the perinuclear space, and the features of the nucleolus. Whereas the nucleo-cytoplasmic ratio and the size of the nucleus become smaller from plasmablast to proplasmacyte to plasma cell, the perinuclear space gets larger. The initially oval nucleus becomes rounder and shifts to a more and more eccentric position. The chromatin gets coarser. All cells of the plasma cell series have a strongly basophilic cytoplasm (Fig. 3, *5*, *6*, *7*).

The position and form of the nucleolus is a key in the distinction between plasmablasts and *basophil stem cells* (immunoblasts) (Fig. 3, *4*). The nucleus of basophil stem cells contains eccentric nucleoli which are often connected to the nuclear membrane. Sometimes plasmablasts have a small perinuclear space and they are in general somewhat smaller than basophil stem cells. The cytoplasm of the latter generally contains a large number of vacuoles.

All of these cell classes (small, large, and cleaved lymphocytes, basophil stem cells, and plasma cells and their precursors) were significantly more frequent in the transplants than in the normal kidneys (Fig. 4).

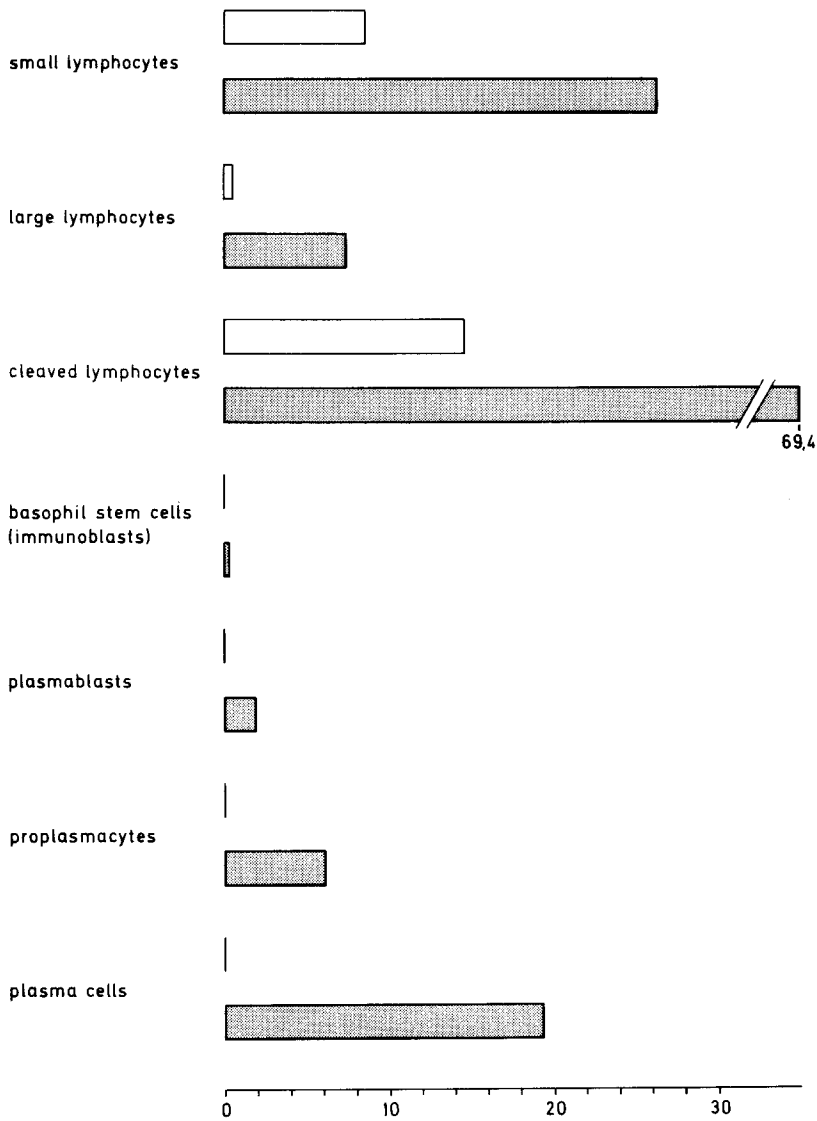


Fig. 4. Arithmetic averages of the relative densities of the immunocompetent cells. The white oblongs denote the values for the reference kidneys, the gray oblongs for the grafts

5. The average relative density of *small lymphocytes* was 26.28 in the transplanted kidneys but only 8.55 in the reference organs. The rank sum of the smaller, group i.e. the controls, was $T = 14$. This means a significant difference between the relative densities of small lymphocytes in the controls and grafts. The probability of error was less than 10% ($p < 0.10$).

6. The average relative density of *large lymphocytes* was 7.39 in the grafts, compared to 0.55 in the normal kidneys ($T = 7$; $p < 0.01$).

Table 1. Ratios of the cell densities in the transplants to those in the reference kidneys

| | |
|---|---|
| Neutrophil polymorphonuclear leukocytes | 1.1 |
| Eosinophil polymorphonuclear leukocytes | 6.0 |
| Monocytes | 0.5 |
| Mast cells | 12.5 |
| Small lymphocytes | 3.1 |
| Large lymphocytes | 13.4 |
| Cleaved lymphocytes | 4.8 |
| Basophil stem cells | $\infty$ |
| Plasmablasts | $\infty$ |
| Proplasmacytes | $\infty$ |
| Plasma cells | $\infty$ |
| Total cell density | 4.4 |

7. The difference for the most frequent of these cell classes, the *cleaved lymphocytes*, was also significant (average relative density in the grafts: 69.4, in the reference kidneys: 14.61; $T=7$, $p<0.01$).

8. The rarest infiltrating cells in the kidneys were the *basophil stem cells* (immunoblasts). The average relative density was only 0.35 in the transplants and 0 in the normal kidneys. The differences in the densities were significant ($T=12$; $p<0.05$).

9. *Plasmablasts* were also rare (grafts: 1.97, reference kidneys: 0) and significantly more frequent in the grafts than in the controls ($T=9$; $p<0.02$).

10. The average relative density of *proplasmacytes* was 6.13 in the grafts and 0 in the reference kidneys ($T=7.5$; $p<0.01$).

11. The average relative density of *plasma cells* was 19.29 in the transplants and 0 in the normal kidneys ($T=6$; $p<0.01$).

### *C. Ratio of the Densities for Each Cell Class*

Table 1 shows the ratio of the cell density in the transplants to that in the reference kidneys for each cell class. The most important feature revealed by these ratios is that the relative densities of the basophil stem cells (immunoblasts), plasmablasts, proplasmacytes, and plasma cells were infinitely greater in the transplants than in the controls.

### *D. Correlations between the Densities of Different Cell Classes in the Transplanted Kidneys*

*1. Plasmablasts/Plasma Cells (Fig. 5).* There is a close positive correlation between these two cell classes ($r=+0.9877$, $p<0.01$, $B=100\cdot r^2=97.65$). The equation for the line of regression is

$$R=8.92\ P+1.83$$

where $R$ and $P$ denote the number of plasma cells and plasmablasts respectively.

*2. Plasmablasts/Proplasmacytes (Fig. 6).* The plasmablast and proplasmacyte densities are also linearly and closely correlated ($r=0.9747$, $p<0{,}01$, $B=95.00$).

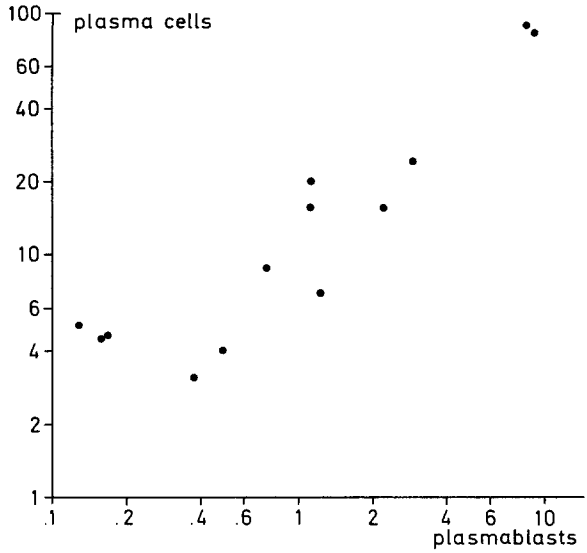


Fig. 5. Correlation between the relative densities of plasmablasts and plasma cells in the grafts shown in a double logarithmic coordinate system ($r = 0.9877$, $p = 0.01$). The values for two cases cannot be demonstrated in this system because they have the coordinates (0,0.05) and (O, 5.5)

The equation for the line of regression is

$$Q = 3.05\ P + 0.16$$

where $Q$ and $P$ denote the number of proplasmacytes and plasmablasts respectively.

*3. Proplasmacytes/Plasma Cells (Fig. 7).* As expected, there is also a close linear positive correlation between these cell densities ($r = 0.9510$, $p < 0.01$, $B = 90.44$). The equation for the line of regression is

$$R = 2.49\ Q + 2.74$$

where $R$ and $Q$ denote the number of plasma cells and proplasmacytes respectively.

### *Further Relations*

Fig. 8 shows that the percentage of plasma cells and precursors out of all immunocompetent cells increase with the density of the plasma cells and precursors. The corresponding linear positive correlation has a probability of error ($p$) of only 0.05. This means that there is definitely a positive correlation even though there is no linear regression.

Moreover, Fig. 9 indicates that the quotients

$$\frac{\text{cleaved lymphocytes}}{\text{plasma cells and precursors}}$$

and

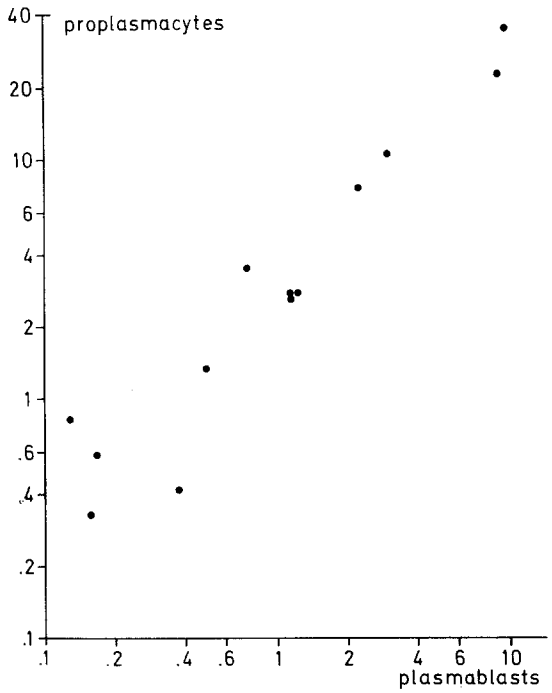


Fig. 6. Correlation between the relative densities of proplasmacytes and plasmablasts shown in a double logarithmic coordinate system ($r = 0.9747$, $p = 0.01$). The values for two cases cannot be demonstrated in this system because they have the coordinates (0, O) and (2.5, O)

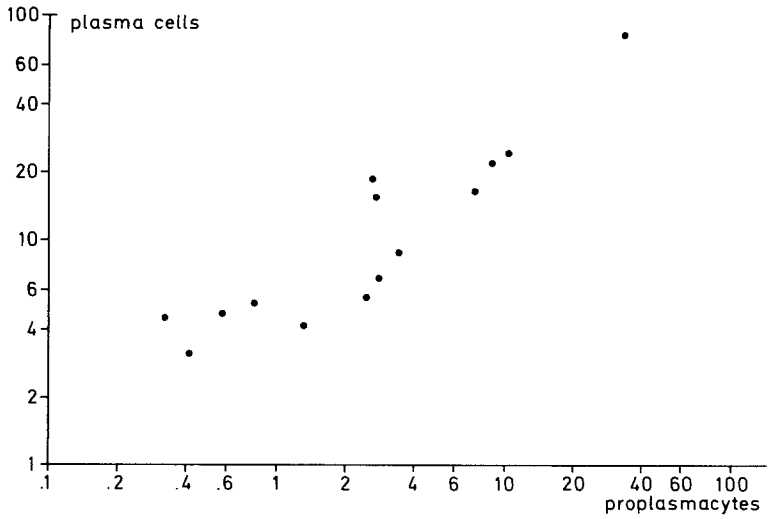


Fig. 7. Correlation beteewn the relative densities of plasma cells and proplasmacytes shown in a double logarithmic coordinate system ($r = 0.9510$, $p = 0.01$). The value for one case cannot be demonstrated in this system because it has the coordinates (0.05, O)

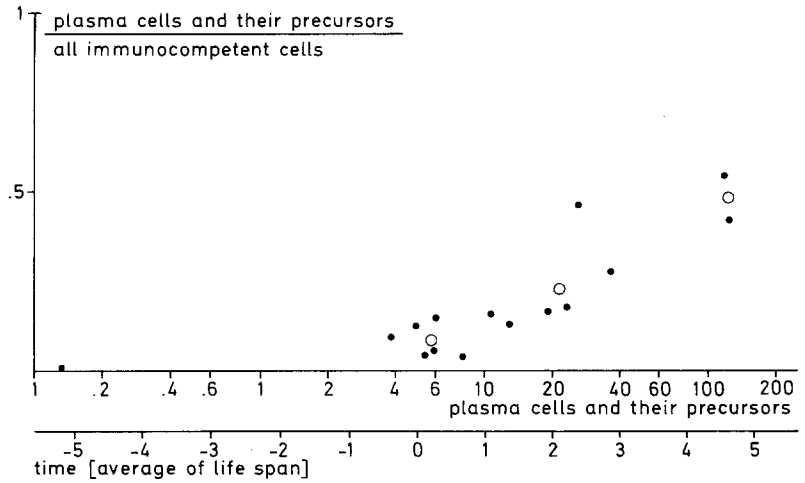


Fig. 8. Increase in the percentage of plasma cells and precursors out of all immunocompetent cells with the increase in the relative density of plasma cells and precursors. ● Values for each case. 0 Average values ($\bar{x}/\bar{y}$) for the classes 1–10, 10–100, and >100

$$\frac{\text{small and large lymphocytes} + \text{basophil stem cells}}{\text{plasma cells and precursors}}$$

approach zero as the density of the cleaved lymphocytes approaches zero.

The sum of these quotients

$$\frac{X+Y}{Z}$$

also approaches zero in the same way ($X$ denotes the relative density of cleaved lymphocytes, $Y$ the sum of the relative densities of small lymphocytes, large lymphocytes, and basophil stem cells, and $Z$ the relative density of plasma cells and precursors). This can be seen immediately in the diagram, since the distance between the first two quotients is equivalent to their sum.

## Discussion

The cellular infiltration which develops in the chronic rejection of human kidney homotransplants has several characteristic features. As our results show, the infiltrating cells may be divided into two groups:

1) Cell classes for which there is no significant difference between the average relative cell densities in the transplants and reference kidneys. This group includes neutrophil and eosinophil polymorphonuclear leukocytes, monocytes, and mast cells. It should be mentioned, however, that the average of the relative densities of eosinophils and mast cells in the grafts was strikingly higher than that for the normal group, but that there was a great spread between the single values from case to case. There is evidence that only mast cells have receptors for IgE (Perelmutter *et al.*, 1972; Gillman, Haddad, 1972). Therefore, the increase in the number

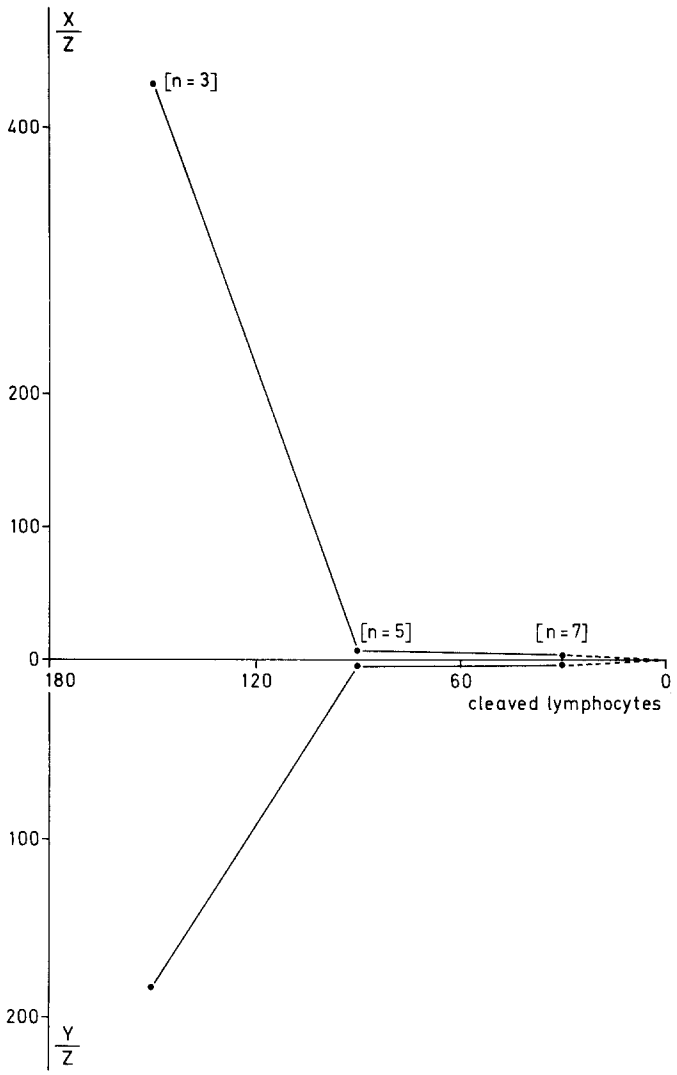


Fig. 9. The relation between the relative density of cleaved lymphocytes and two ratios: on the top the ratio of the relative density of cleaved lymphocytes ($X$) to that of plasma cells and precursors ($Z$), and on the bottom the ratio of the sum of the relative densities of small and large lymphocytes and basophil stem cells ($Y$) to the relative density of plasma cells and precursors ($Z$)

of mast cells in the grafts suggests that a component of IgE may play a role in the chronic rejection of transplanted human kidneys.

Eosinophils can be attracted chemotactically by the release of histamine (Archer, 1968). They may appeared in greater numbers in the grafts for this reason. However, we did not find a correlation between the densities of mast cells and eosinophils, so the presence of eosinophils in the transplants may have another

explanation. Trentin *et al.* (1971) showed that the eosinophilic granulocyte response to antigen appears to be independent of an increase in antibody. They suggested that a thymic derived mononuclear cell population is necessary for an optimal eosinophil response to antigen.

2) Immunocompetent cells. This group includes small and large noncleaved lymphocytes, cleaved lymphocytes, basophil stem cells (immunoblasts), and plasma cells and precursors. They were significantly more frequent in the grafts than in the reference kidneys. A further subdivision can be made, however: the densities of the plasma cells and precursors and of the basophil stem cells were infinitely greater in the grafts than in the normal kidneys, whereas the corresponding ratio of the other immunocompetent cells hardly differed from that of the polymorphonuclear leukocytes, monocytes, and mast cells.

Since we found not only plasma cells but also precursors in the grafts, the question arises as to whether the plasma cells originated from the precursors through proliferation in the kidney itself. If this is the case, one would expect a strict linear correlation between the densities of these cell classes. The inclinations of the lines of regression would have to be integral positive powers of 2 or a sum of such powers of the form $2^k+2^{k+1}+\ldots 2^{k+q}$, since in each case two cells grow out of each cell division. From counting cells and mitotic figures in lymph nodes Sainte-Marie (1966) concluded that the population of plasmablasts is composed of 4 generations, the population of proplasmacytes of 2 following generations, and the population of plasma cells of 2 more generations. The plasmablast density would therefore be $2^0+2^1+2^2+2^3=15$, compared to $2^4+2^5=48$ for the proplasmacytes and $2^6+2^7=192$ for the plasma cells. The ratios between these values would be 1:3.2:12.8. The inclination of the line of regression between plasmablasts and proplasmacytes deviates negligibly from these theoretical values. That between plasmablasts and plasma cells, however, was determined to be 8.92, compared to the theoretical value of 12.8. This deviation is explained largely by the destruction of mature plasma cells in the transplants, which could be seen microscopically. The ratios between the cell densities may also have been influenced by the different life spans of the various generations.

However, the close positive linear correlation between plasmablasts, proplasmacytes, and plasma cells can hardly be explained in any other way than by assuming that proliferation of the plasma cell line occurred in the grafts themselves. Therefore the marked proliferation of mononuclear lymphoid cells in grafts seen by Lindquist *et al.* (1969) was probably a proliferation of plasma cells and precursors. Pederson and Morris (1970) also concluded that the transformation and proliferation of lymphoid cells involved in the rejection process occur in the transplant itself.

A further anlysis of the cell counts shows that the percentage of plasma cells and precursors out of all immunocompetent cells increases with the density of the former. Assuming that plasma cells develop through proliferation of their precursors in the grafts themselves, the number of plasma cells and precursors per 10 microscopic fields would double after the average life span of all plasma cell precursors capable of division. Therefore the increase in the percentage of this cell lineage out of all immunocompetent cells would be a function of time (Fig. 8).

The reciprocal value of this percentage may be written like this:

$$\frac{V}{Z} = \frac{X+Y+Z}{Z} = \frac{X+Y}{Z} + \frac{Z}{Z} = \frac{X+Y}{Z} + 1$$

where $V$ denotes the relative density of all immunocompetent cells, $Z$ the relative density of cleaved lymphocytes, and $Y$ the sum of the relative densities of small and large lymphocytes and basophil stem cells (immunoblasts).

It follows that the reciprocal value $V/Z$ approaches one if $(X + Y)/Z$ approaches zero (Fig. 9).

Therefore the relative number of plasma cells and precursors out of all immunocompetent cells also approaches one. This would mean that the composition of the immunocompetent cell population shows a trend toward a pure population of plasma cells.

What do our results mean with respect to the rejection of grafts, in particular the chronic rejection of human kidney transplants? Unfortunately, it is not possible to determine the exact time of rejection in any given clinical situation. If this point is reached before plasma cells occur in the grafts, then it could be that the plasma cell reaction with all of its consequences is merely the reaction of an "innocent bystander". Otherwise we suspect that the plasma cell should be looked upon as the true "effector cell" among the immunocompetent cells, at least in the chronic rejection of human kidney transplants. This would agree well with the findings of Pederson and Morris (1974), which indicated that in sheep the terminal stage of primary allograft rejection is mediated by humoral antibodies.

However, this interpretation of our results contradicts the opinion of many other authors, who thought that $T$-lymphocytes are generally the effector cells in the rejection of grafts (Roitt *et al.*, 1969; Fischer *et al.*, 1970).

On the other hand, in a review of the studies of the role of $T$-lymphocytes Cerottini and Brunner (1973) admitted that "the results, however, did not exclude the possibility, although unlikely, that the immune $T$ cells functioned as helper cells for the production of alloantibody which caused rejection in the presence of complement or non-$T$-effector-cells".

There is further support for our interpretation. Perlman *et al.* (1974), for instance, found no evidence that $T$-lymphocytes were the effector cells in an in-vitro-system of cell-mediated cytolysis. Grant *et al.* (1970) showed that the lysis of L-5178 mouse lymphoma cells after addition of sensitized immunocompetent sheep cells was mediated by humoral antibodies and complement. Baldemus *et al.* (1973) even succeeded in effecting a rejection of skin allografts in mice solely by humoral antibodies.

A further point for discussion is the role of the cleaved lymphocytes, which were the most frequent of the cells we counted in the grafts. The term cleaved follicle center cell was used by Lukes and Collins (1973) for the cell which had been described as a germinocyte by Lennert (1964) and Mori and Lennert (1969) which belongs to the bursal equivalent or bone marrow derived or "$B$"-cell system. Germinal centers are sites of proliferation of $B$-cells which give rise to precursors of the plasma cell series (Lennert *et al.*, 1967; Veldman, 1970; Nieuwenhuis, Keuning 1974; Nieuwenhuis *et al.*, 1974). The question arises as to whether the cleaved

lymphocytes are identical to germinocytes or whether they are perhaps related to the *T*-cell system. This possibility has to be considered since they show some similarities to a neoplastic cell of the *T*-cell system, the Sézary cell (Lutzner, Jordan, 1968; Lutzner *et al.*, 1973). So it is not yet clear whether the cleaved lymphocytes which are involved in the rejection of human kidney transplants are *B*-lymphocytes — perhaps antibody forming cell precursors — or *T*-cells — perhaps *T*-helper cells.

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