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Application of flow cytometry in clinical renal transplantation

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Abstract. Flow cytometry (FC) may be considered as a fundamental technique in studying cell biology and pathology. It combines the quantitative character of biochemical methods with the multiparametric capacities of microscope analysis in a high-precision process for rapid analysis of individual cell characteristics. Three original FC techniques routinely applied in the field of renal transplantation are reported in the present study. They concern the donor–recipient cross-match test, the morphological analysis of urinary sediment and the modulation of the density of various membrane antigens on the lymphocyte surface. A common factor underlies all these methods: they aim to provide the physician with a reliable diagnostic tool in clinical renal transplantation.

Key words: Flow cytometry – Renal transplant – Lymphocytes

Flow cytometry (FC) today may be considered as a fundamental technique in studying the biology and pathology of cells. Its development and application in biological research and clinical diagnosis respresents a successful example of multidisciplinary 'hybrid technology', based on the confluence of advanced technologies such as radiation physics, computer science, fluorochrome chemistry, cytochemical staining and monoclonal antibody production.

FC has introduced new vistas in the identification and characterization of cell populations [7], combining the quantitative character of biochemical methods with the multiparametric capacities of microscopic analysis in a high-precision technique for rapid analysis of individual cell characteristics [8]. These qualities suggest that FC may be of the greatest use in organ transplantation.

The application of flow cytometry in the field of renal transplantation has hitherto largely consisted of the immunological monitoring of patients performed through lymphocyte subset typing by means of fluoresceinated monoclonal antibodies [2, 4]. The technical performance of the last-generation instruments (both analysers and sorters), the increased scientific knowledge and technical experience, and the range of sophisticated diagnostic reagents have progressively extended the scope of flow cytometry techniques to the clinical management of transplanted patients.

This report presents a short review of three FC techniques (designed by us) which are routinely applied at the Nephrology, Dialysis and Transplantation Institute of Bologna University. They concern different areas from among the extremely wide possibilities afforded by FC, but are unified by a common factor: they aim to provide the physician with a diagnostic tool when facing a clinical problem in renal transplantation.

Flow cytometry evaluation of the pretransplant donor–recipient cross-match test in renal transplantation

In renal transplantation the cross-match test (CM) evaluates the existence and degree of presensitization of a potential graft recipient against the kidney donor. It is generally accepted that the presence of preformed antibodies (positive CM) represents an absolute contraindication to transplantation.

The standard optically based method, a complement-dependent cytotoxic assay [12], may sometimes be not completely reliable, even in experienced centres, for purely technical reasons: (1) the difficulty in detecting weak positive reactions (false negative results); (2) a high number of dead or contaminating cells present under light microscopy observation (false positive results); and (3) the possibility of operator errors due to test evaluations not being sufficiently standardized.

In order to optimize pretransplant CM evaluation, we combined the standard light microscopy method with an innovatory FC technique based on cytometrical analysis of the cytotoxic assay itself [13]. Figures 1 and 2 show the

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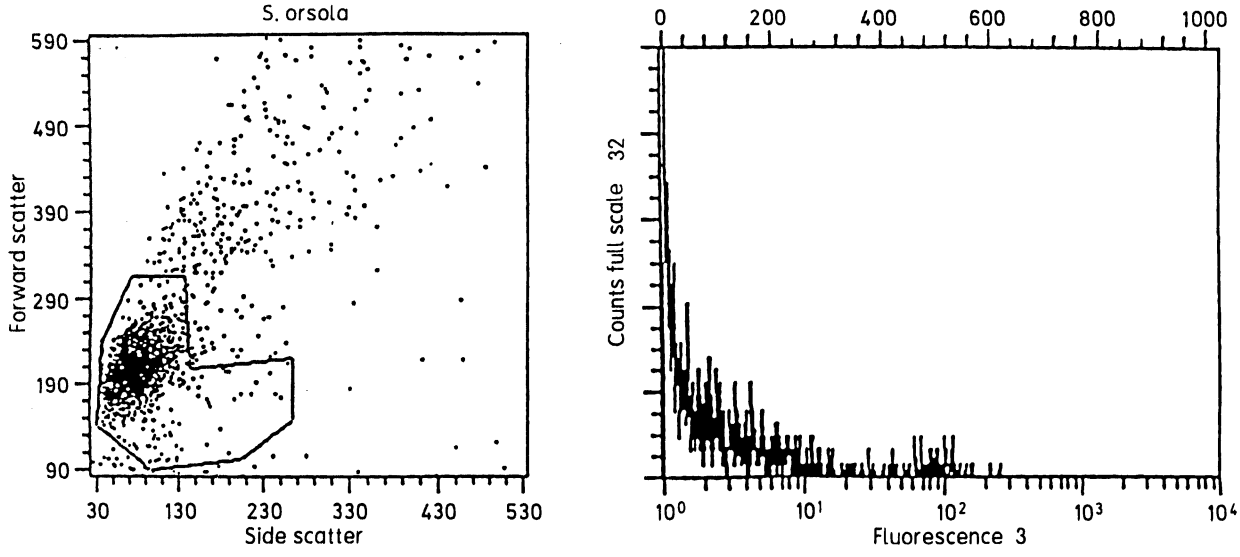


Fig. 1. Cytogram *(left)* and histogram *(right)* of a negative CM test

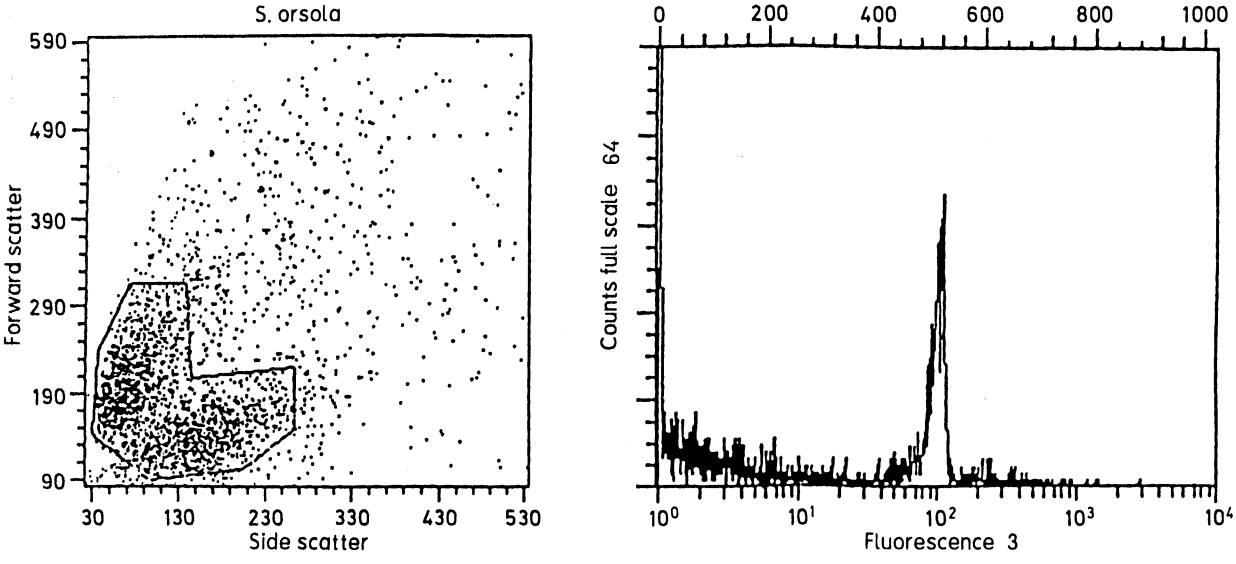


Fig. 2. Cytogram *(left)* and histogram *(right)* of a positive CM test

cytogram and the histogram of negative and positive samples obtained on a FACSCAN Flow Cytometer (Becton-Dickinson). Of note is the unusual shape of the positive dead lymphocytes which fall into the lower right lobe of the established gate window (Fig. 2). The study included 5185 cross-match tests performed over 24 months at the Institute of Nephrology, Bologna University, using lymphocytes from 62 consecutive kidney donors and sera from 431 candidates for transplantation.

Out of the 5185 CM examined by both techniques, 1171 tests (22.6%) proved positive with light microscopy, while 1504 (29.0%) were positive with FC (Fig. 3). The difference is statistically significant ($P < 0.001$). Comparing the two techniques, 719 samples out of the 5185 (13.87%) received different evaluations: 526 CM were positive with FC but negative with light microscopy examination ($P < 0.001$), while 193 proved negative with FC and positive with light microscopy (Fig. 4).

Figure 5 shows the correlation between the results obtained by this technique and graft survival. After 1 month graft survival was significantly higher ($P < 0.02$) in patients for whom CM was evaluated by both techniques, than in a second patient group, in which CM was examined only by light microscopy; after 3 months this difference was reduced and tended to disappear during the first year.

Compared with light microscopy the technical advantages were:

1. Each CM test was assessed on a high number of lymphocytes.
2. Sample evaluation was computerized.
3. The threshold between negative and positive was clearly identified.
4. Detection of weak positive reactions was enhanced.
5. False positive reactions due to insufficient purification of the sample were avoided.

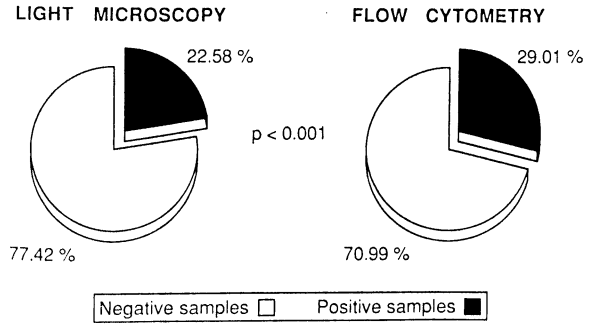


Fig. 3. Percent distribution of positive and negative pattern in 5185 cross-match tests. Light microscopy *(left)* and flow cytometry *(right)*

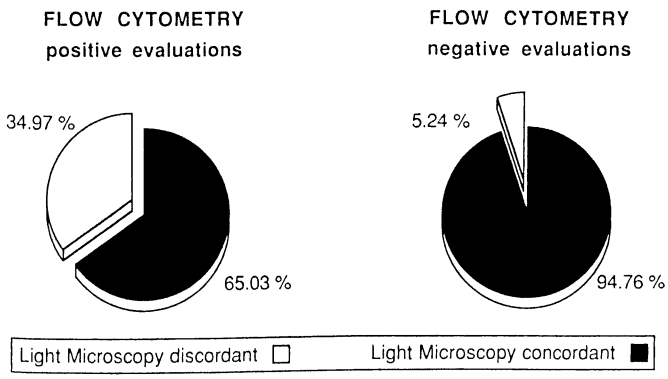


Fig. 4. Comparison of flow cytometry and light microscopy evaluation in 5185 cross-match tests

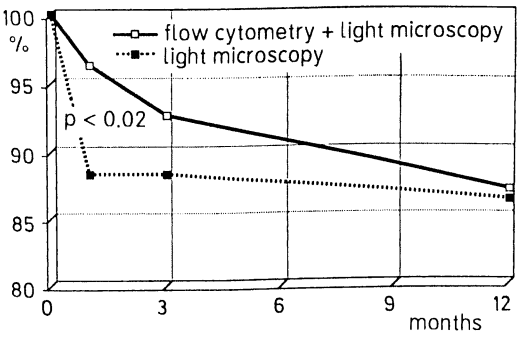


Fig. 5. Graft survival and clinical outcome in patients with negative CM test determined by both flow cytometry and light microscopy evaluation ($n = 51$) or by the standard microscopic technique only ($n = 54$)

6. The risk of selecting candidates with donor presensitization (false negative CM) was reduced.

In short, the cross-match technique we have developed exploits the same biological reaction (i.e. complement-dependent cytotoxicity) as is used in standard light microscopy assays. The advantage of our method over this still-accepted technique for donor–recipient cross-match testing in clinical transplantation is that it increases the sensitivity of the reading, thus reducing the possibility of error.

Clinical results support the validity of this technical improvement, showing that the incidence of primary renal non-function and early graft loss could be reduced. On this basis the new FC technique we have developed seems to be a reliable and helpful assay for pretransplant investigations in renal transplantation, representing an interesting addition or even an alternative to light microscopy cross-match evaluation. A detailed report has been published elsewhere [13].

Urinary cytology

The value of exfoliative urinary cytology for the diagnosis of different pathological conditions in renal transplantation has been suggested by various investigators [11]. In particular, the presence of lymphocytes has been suggested as an indicator of acute rejection episodes [6]. This method, however, has not gained wide acceptance, very likely because of the difficulty in obtaining a reliable identification of the different cells by means of standard staining techniques.

FC may make a significant contribution to this 'pure morphology' approach thanks to its powerful combination of light microscopy examination characteristics, such as multiparametric analysis, and the computerized quantitative evaluation of single cellular elements [10, 15]. The method we designed was aimed at analysing urinary sediment cells in renal transplanted patients in order to define the morphological features of the various populations, i.e. lymphocytes, monocytes/macrophages and granulocytes, involved in the immune response.

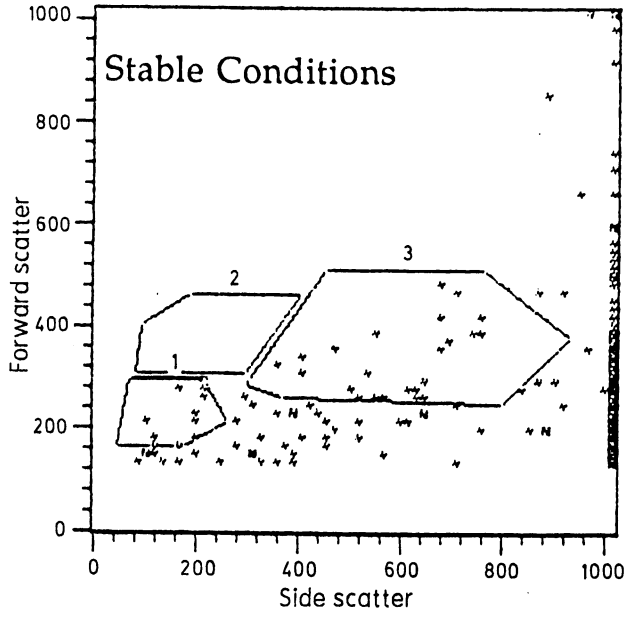
We used FC analysis of the urinary sediment in a wide-ranging study involving 233 urine sediment samples from 173 renal transplanted patients, selected on the basis of clinical condition:

A. normal renal function, without clinical or laboratory evidence of bacterial infection
B. acute rejection
C. bacterial infection of the urinary tract
D. post-transplant tubular necrosis diagnosed by clinical signs (oliguria) or laboratory investigation (creatinine clearance lower than 10 ml/min)
E. laboratory signs of cyclosporine cytotoxicity.

Urine sample preparation and instrumental technical details have been reported elsewhere [10].

Results showed that the number and the percentage distribution of the identified cell populations in the patient groups depended on clinical condition. As far as lymphocyte and monocyte numbers were concerned the 'acute rejection' group showed the highest value; similarly polymorphs and debris were typical, respectively, of the 'bacterial infection' and the 'acute tubular necrosis' group.

Figure 6 reproduces urinary sediment cytograms which refer to various clinical conditions: patients with stable renal function had a sediment with a low cell count, acute rejection was characterized by significant lymphocyturia (associated with monocyturia in the case of vascular involvement), while polymorphs and debris predominated in urinary tract infections. During post-transplant tubular necrosis the noteworthy finding concerns the debris which assumes a 'high scatter' pattern, i.e. high density and large size particles, while, interestingly, in patients with clinical or laboratory signs of cyclosporine cytotoxicity the debris


Stable Conditions
Forward scatter
Side scatter
0
200
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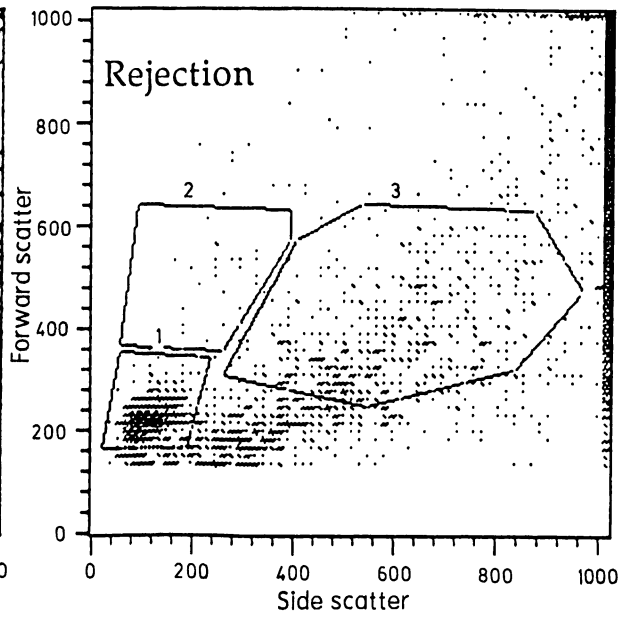
Rejection
Forward scatter
Side scatter
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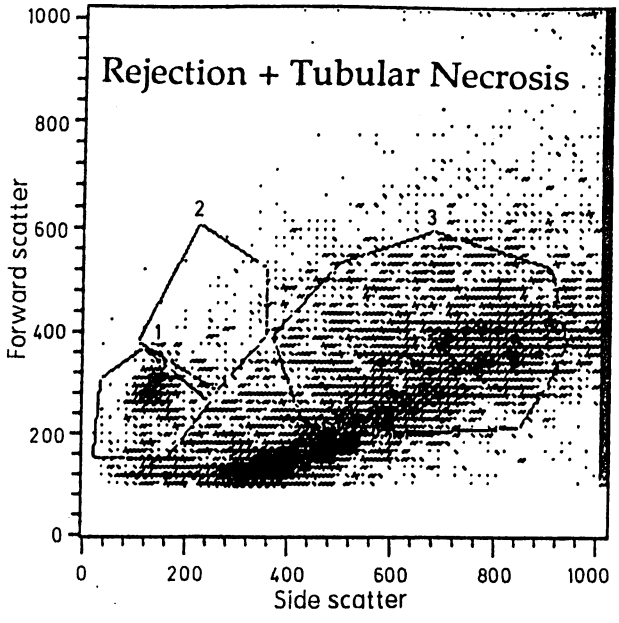
Rejection + Tubular Necrosis
Forward scatter
Side scatter
0
200
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800
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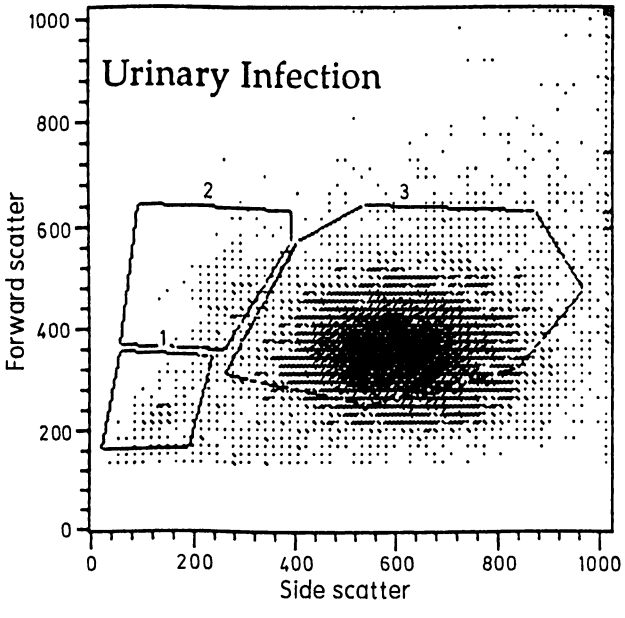
3


Urinary Infection
Forward scatter
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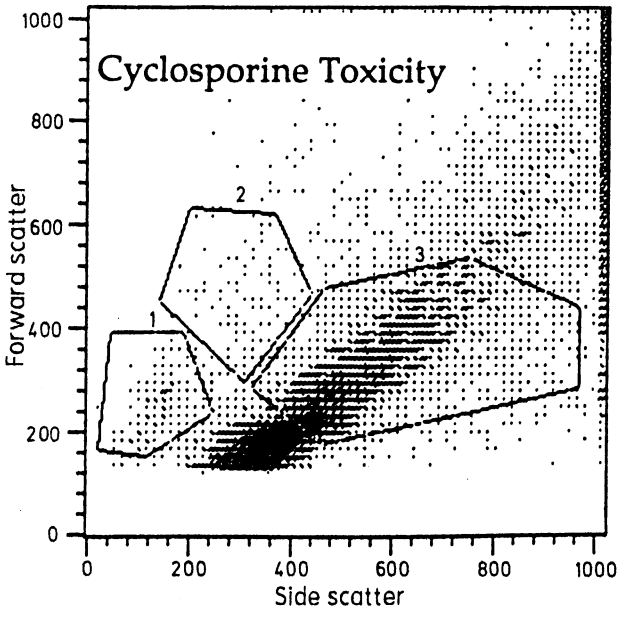
Cyclosporine Toxicity
Forward scatter
Side scatter
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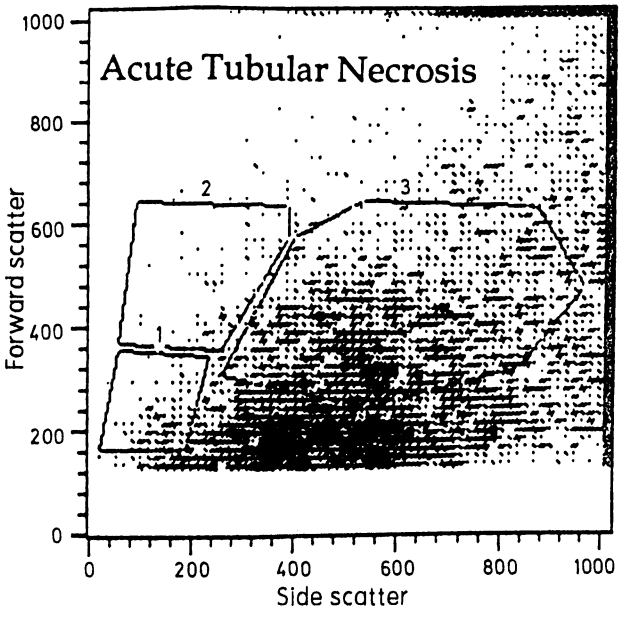
Acute Tubular Necrosis
Forward scatter
Side scatter
0
200
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shows a 'low scatter' pattern, i.e. low density and small size particles.

Cytometric urine analysis appears of use as a first-step investigation both in the diagnostic approach to transplant patients and in examining the real morphological situation of the allograft. We hope that in future this test will come to be considered as the morphological equivalent to the creatinine clearance test.

Evaluation of surface antigen expression on lymphocyte membranes

The combined use of FC and monoclonal antibodies enables one to perform a quantitative analysis of the density of the surface antigens expressed on lymphocyte membranes [9]. In order to evaluate whether this determination could be related to the degree of immunological activity, we examined 60 renal transplant patients, chronically treated with cyclosporine and steroids, and 15 normal subjects as a control group.

Isolated peripheral blood lymphocytes were reacted with monoclonal antibodies specific for the antigens of the first and second class of the major histocompatibility complex (MHC), for $\beta 2$-microglobulin and for the alpha-beta T-cell receptor (TCR) by means of an indirect immunofluorescence technique. A FACSCAN Flow Cytometer (Becton-Dickinson) was used to analyse the mean intensity of fluorescence which is directly related to the number of molecules expressed on the cell surface.

The quantitative expression of MHC antigens (class I and II) and TCR in transplant recipients in a stable clinical condition (quiescence level) was significantly lower (Figs. 7 and 8) than in normal subjects ($P < 0.01$). During episodes of high immunological activity, such as acute rejection episodes and viral infection, TCR (Fig. 8) and MHC class II antigen expression (Fig. 9) showed a significant increase ($P < 0.01$ vs quiescence level). Patients treated with aggressive immunosuppressive therapy (antilymphocyte globulin and OKT3 monoclonal antibody intravenously) showed a significant reduction in antigen expression (Figs. 8 and 9) regarding both MHC antigens and TCR ($P < 0.01$ vs quiescence levels).

The main observations emerging from these data are:

1. After renal transplantation the intensity of fluorescence staining of circulating lymphocytes is poor, depending on the reduced density of surface antigens, very likely related to immunosuppressive therapy [5].
2. There is probably a close relationship between clinical phases of high reactivity against the allograft, manifested as acute rejection episodes, and an increased density of TCR and MHC class II antigens [3].
3. The same findings were also observed during CMV infection. In this condition the increased antigen expression reflects an activation phase of T lymphocytes, presumably committed to virus-infected cells [16].

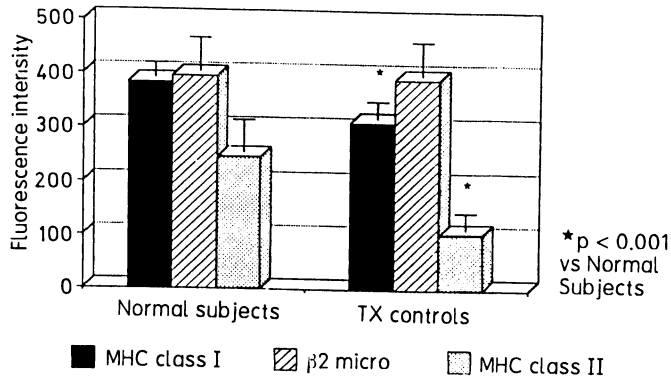

Fig. 7. Quantitative evaluation of MHC antigens (class I and II) and $\beta 2$ microglobulin expression on peripheral blood lymphocytes in renal transplantation

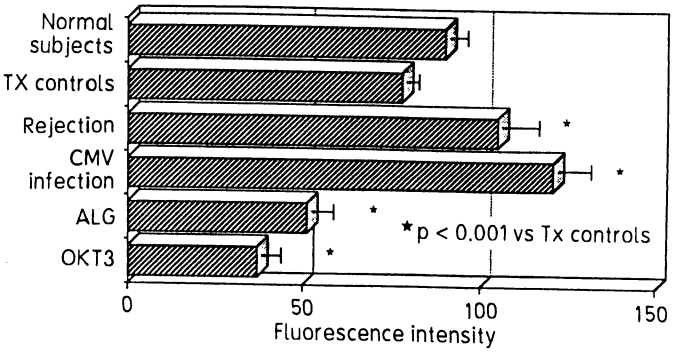

Fig. 8. Quantitative evaluation of TCR expression on peripheral blood lymphocytes in renal transplantation

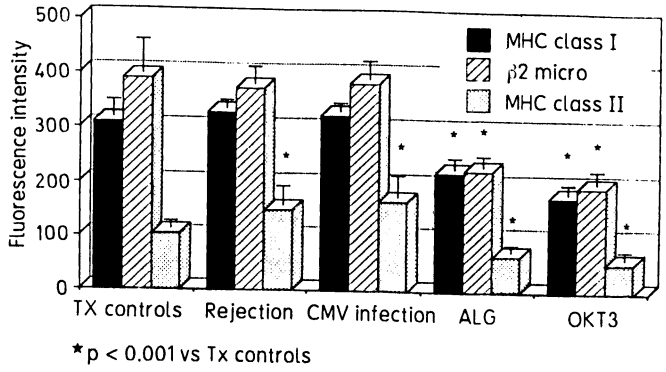

Fig. 9. Up- and down-regulation of MHC antigen expression on peripheral blood lymphocytes in various clinical conditions in renal transplantation

4. The significant reduction in antigen expression in patients treated with ALG is very likely related to the effect of antilymphocyte globulin which unselectively recognizes all lymphocyte structures [11], while the marked alterations which occur during OKT3 treatment depend on rearrangements in the molecular surface structure, such as internalization and modulated re-expression, which take place during treatment [1].

The significant correlations we found between patients with differing clinical conditions and the density of various antigens and receptors expressed on the lymphocyte membrane suggest that the quantitative evaluation of

Fig. 6. Renal transplantation. Urinary cytograms in various clinical conditions

these parameters may enable us to go beyond simple lymphocyte subset typing, providing the physician with useful information on lymphocyte functional activity.

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