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Research Article

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Alterations in T and B Lymphocytes in Heart Transplant Patients Early and Late Postoperatively

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ABSTRACT Alterations in the percent and absolute number of thymus-derived (T) and bursa-equivalent (B) lymphocytes in peripheral blood were followed in 10 patients treated with antithymocyte globulin, prednisone, and azathioprine after cardiac transplantation. During the 1st postoperative wk the percent of T cells dropped below 10% in almost all cases (normal range, 65–91%) with a concomitant rise in the percent of B cells. However, the absolute T- and B-cell counts were both markedly depressed (< 200 cells/mm^3). During the 7-wk postoperative period the percent of T cells rose to 45–60% and the absolute count rose from 100 to 350 cells/mm^3 (normal range, 1,092–2,400 cells/mm^3). Although the percent of B cells was elevated (35–50%) during this period, the absolute B-cell count remained below the range of normals (268–640 cells/mm^3). Follow-up of long-term survivors (3–60 mo postoperative) showed a continued marked T (467 cells/mm^3) and B (95 cells/mm^3) lymphocytopenia. Chronological relationships between the percent and absolute T-cell count and episodes of graft rejection in individual patients are discussed as possible adjuncts in the prediction of rejection crises.

INTRODUCTION

Prolonged survival of renal and cardiac allografts in man has been achieved using a variety of immunosuppressive regimens (1–4). Determination of the dosage, time course, and type of immunosuppressive agents has been mainly empirical and related to the presence or absence of graft rejection. Few immunological parameters have been used to assess the impact of immunosuppression between rejection episodes or to predict the onset of rejection crises.

Thymus-derived (T) lymphocytes play a central role in acute rejection of organ transplants (5–7). Recently, several techniques for the identification and quantitation of T lymphocytes in human peripheral blood have been reported. The present study was carried out to determine percent and absolute number of T cells in the peripheral blood of patients treated with a combination of antithymocyte globulin (ATG),[1] prednisone, and azathioprine after cardiac transplantation. It was expected that the level of T cells in the peripheral blood might be a useful aid in evaluating the efficacy of immunosuppressive treatment and in predicting rejection episodes. In addition, the effects of chronic immunosuppressive therapy on T and bursa-equivalent (B) cells were compared to determine the target cell specificity of the treatment.

METHODS

Patients. 10 patients undergoing cardiac transplantation because of advanced incapacitating cardiac disease were studied. Six patients presented severe coronary artery disease complicated by irremediable left ventricular dysfunction, and four patients presented idiopathic cardiomyopathy. Nine of the patients were men, and the mean patient age was 36 yr (range, 15–49 yr). Preoperative medical treatment included digoxin and various diuretic drugs in all patients. Postoperative drug therapy (excluding immunosuppressive agents) included diuretics, warfarin sodium, and dipyridamole. The operative techniques utilized for cardiac transplantation have previously been described in detail (8).

16 of the 21 rejection episodes documented in the group of 10 patients occurred during the first 35 postoperative days. There was one death which occurred on day 48. Patients were discharged from the hospital between postoperative days 45–101, and were not hospitalized thereafter unless a rejection episode intervened.

Diagnosis of acute cardiac graft rejection. Methods for

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[1] *Abbreviations used in this paper:* ATG, antithymocyte globulin, B, bursa equivalent; T, thymus derived.

the diagnosis of cardiac allograft rejection in man have been described previously (9, 10). In brief, the clinical diagnosis of graft rejection was predicated on a decrease, exceeding 20 percent of baseline, in the algebraic sum of QRS voltages in standard electrocardiographic leads I, II, III, V^1, and V^6 or upon the development of a new early or late diastolic gallop rhythm, indicative of decreased ventricular compliance. All episodes of diagnosed and treated graft rejection in the patients included in this report, however, were confirmed histologically by transvenous endomyocardial biopsy (10). Typical morphological features indicative of acute rejection in such biopsy specimens include interstitial and intracellular (myocyte) edema, perivascular and interstitial infiltration with mononuclear cells containing methyl green pyronine-positive cytoplasm, endocardial thickening and cellular infiltration, and occasionally areas of myocyte destruction with fibrous replacement.

Immunosuppression. Immunosuppressive therapy included azathioprine, prednisone, and ATG of rabbit origin in all patients. All recipients received a loading dose of azathioprine, 4–5 mg/kg orally, immediately before operation, and maintenance doses of 2–3 mg/kg per day postoperatively, as determined by the peripheral leukocyte count. Methylprednisolone, 5 mg/kg, was given intraoperatively to all patients, and maintenance oral prednisone treatment was begun on the 1st postoperative day in a dose of 1.5 mg/kg, and gradually decreased to 1.0 mg/kg daily by the end of the 2nd postoperative wk.

Antihuman thymocyte globulin was produced in New Zealand white rabbits by a modification of the method of Davis et al. (11). Immunizing doses consisted of 10^9 fresh human thymocytes injected a month apart. The first was given in complete Freund's adjuvant in multiple cutaneous sites and the second dose was given intravenously. Bleeding of a pool of 20 rabbits was performed 1 wk later and the separated serum was precipitated with ammonium sulfate. The gamma globulin content of the pooled preparations ranged from 63–80% of total protein. Rosette inhibition titers (12) were determined for each pool and averaged 1/40,000. Dosage of ATG was regulated according to the rosette inhibition titer of the preparation used; 1-ml of a 50 mg protein/ml globulin preparation was considered to contain a number of "units" equal to the reciprocal of the rosette inhibition titer (RIT units). Each dose, administered intramuscularly, consisted of 4,000 U/kg. The schedule of administration utilized in all patients consisted of six initial doses on postoperative days 0, 1, 2, 4, 6, and 8. Thereafter, ATG was administered only during some of the diagnosed acute rejection episodes in courses of six doses (daily for 3 days, then every other day for three doses).

Assay for T cells in the peripheral blood. T lymphocytes were identified by an in vitro cytotoxicity test using a specific anti-T-cell serum (13). Anti-T-cell serum was prepared by immunizing goats with thymus cells obtained from children undergoing cardiac surgery. The crude antithymocyte serum was extensively absorbed with human erythrocytes and a lymphoblastoid cell line which carries surface immunoglobulin as judged by immunofluorescent staining. We have previously shown that these absorbed antisera kill less than 10% of a suspension of pure Ig-bearing human peripheral blood lymphocytes obtained by automatic separation on a fluorescence activated cell sorter (13). In addition, the specificity of each batch of antiserum is standardized by measuring the percent kill of pure suspensions of E-rosette forming and nonrosette forming cells from normal donors. E-rosette formation is performed according to Bentwich et al. (14), and rosettes are separated on a Ficoll-Hypaque gradient. Specific antisera kill greater than 95% of rosette forming cells, and less than 10% of nonrosette forming cells. The latter cells are made up of a mixture of Ig-bearing small cells (60–85%), monocytes, and null cells as judged by techniques described below.

The cytotoxic assay is performed in 4×50-mm glass tubes. 50 μl of anti-T-cell serum or normal goat serum diluted 1:4 in medium 199 (Microbiological Associates, Inc., Bethesda, Md.) with 5% fetal calf serum are added to 25 μl of a suspension of 1.25×10^5 peripheral blood lymphocytes. After incubation at room temperature, guinea pig complement is added, and the percent of dead cells is determined thereafter by trypan blue exclusion. The percent of T cells is calculated from the cytotoxic index (C. I. = percent of experimental cells killed − percent of control cells killed/100 − percent of control cells killed) after adjustment for the percent monocyte contamination. The absolute T-cell count is calculated by multiplying the percent of T cells by the absolute lymphocyte count (determined by total leukocyte and differential counts). Nonspecific killing of normal cells by normal goat serum and complement is less than 5%.

Assays for B cells. Peripheral blood B lymphocytes were identified by staining for surface Ig using a fluorescein-conjugated rabbit antihuman Ig antiserum (Antibodies Inc., Davis, Calif.) as described previously (13). The percent of positive cells is determined by examining approximately 200 cells by immunofluorescence microscopy. Only small cells were examined, since these are free of monocytes as judged by histochemical staining.

Histochemical staining of peripheral blood monocytes. Monocyte contamination of Ficoll purified peripheral blood cells was determined by staining with α-naphthol acetate according to Yam et al. (15). α-naphthol acetate stains nonspecific esterase present in monocytes, but does not stain lymphoid or myeloid cells (15).

Blood samples. Blood samples (10–20-ml heparinized blood) were assayed two to three times per wk for each patient. Data is presented for 7 wk postoperatively, since many patients were discharged from the hospital after that time. Despite severe lymphopenia in most patients, 100–400 cells were counted on each occasion to determine the percent of T cells.

RESULTS

Percent and absolute number of T and B cells in normal controls and in patients with congestive heart failure. Table I shows the mean percent and absolute levels of T cells and B cells in normal laboratory personnel at the Stanford University Medical Center. The mean absolute counts were 1,600 T cells/mm^3 and 407 B cells/mm^3. The mean percentages were 77% T cells and 20% B cells. Detailed values for each individual have been reported previously (16). The sum of the percent of T and B cells varied between 86–107% in this group.

Table II shows data for seven patients with congestive heart failure treated with the same preoperative cardiac medications (digitalis and diuretics) as the heart transplant recipients. Although the mean percent (75%) and absolute T-cell count (1,681/mm^3) do not differ significantly from the mean of normals, four of

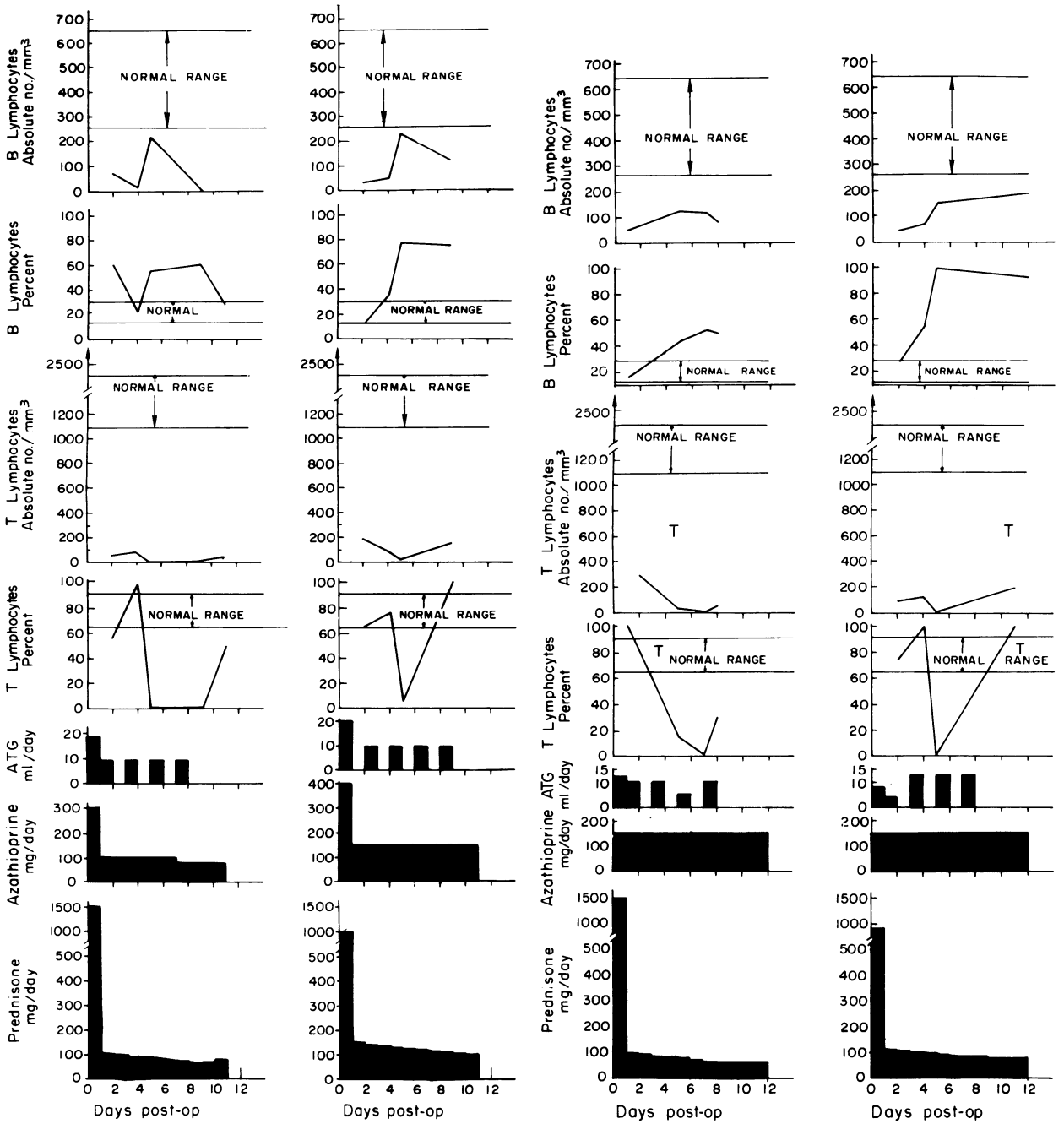

FIGURE 1 Percent and absolute T- and B-cell counts during the first 12 postoperative days in four patients.

seven patients showed an absolute T-cell count below the normal range. The mean percent (14%) and absolute number (358/mm³) of B cells were significantly below normal ($P < 0.01$), as judged by the Student's t test for unpaired data.

Alterations in T and B lymphocytes during immediate postoperative period. Fig. 1 shows the alterations in the percent and absolute number of peripheral blood T and B cells during the first 12 days after transplantation in 4 patients which are representative of the group of 10. In each case, the percent of T cells fell below 10% during the 1st wk of therapy. An abrupt rise in the percent of T cells occurred during the 2nd wk, frequently rebounding to 100%. In almost all cases, how-

TABLE I

T and B Lymphocytes in Normal Donors

Cytotoxicity	Monocytes and myeloid cells	T lymphocytes	B lymphocytes	Absolute lymphocytes	Absolute T lymphocytes	Absolute B lymphocytes
%	%	%	%	*counts/mm³*	*counts/mm³*	*counts/mm³*
64±1.6*	17±1.2	77±1.5	20±1.1	2,038±73	1,600±76	407±25
49–80‡	10–30	65–91	13–30	1,400–2,700	1,092–2,400	268–640

* Mean±SE of 22 donors.

‡ Range.

ever, the absolute T-cell count remained under 200 cells/mm³ during the first 2 wk, despite the rise in the percent of T cells.

A rise in the percent of B cells occurred simultaneously with the fall in the percent of T cells (Fig. 1). However, the percent of B cells remained elevated in some cases while the percent of T cells rebounded during the 2nd week. The sum of the percent of T and B cells in these cases was sometimes greater than 150%. In contrast, the sum of the percent of T and B cells in normal controls never exceeded 105%.

The markedly elevated sum of the percent of T and B cells noted in some patients was transient (3–5 days) and infrequent, but appeared to be related to concurrent or recent treatment with ATG. A possible explanation for this finding is that treatment with ATG results in the coating of some T cells with human immunoglobulin. The latter cells would be spuriously identified as B cells since they bear human Ig on their surface. To test this point, peripheral blood lymphocytes from two normal controls were typed for T and B cells before and after incubation for 1 h at 37°C in vitro with serum from two heart transplant patients receiving ATG. Table III shows that in three out of four cases the percent of B cells rose substantially after incubation, and the sum of the percent of T and B cells exceeded 115%. Measured percent of T cells was not significantly different.

Although the percent of B lymphocytes was elevated during the first 2 wk, the absolute B lymphocyte count was depressed in almost all cases (< 200 cells/mm³) (Fig. 1). Combined early postoperative therapy, therefore, produces both T- and B-cell lymphopenia, and affects the T cells proportionately more severely than the B cells.

Changes in the percent and absolute number of T cells in the peripheral blood during the 7-wk postoperative period. Fig. 2 shows the weekly mean percent of T cells in the peripheral blood of the 10 patients during the 7-wk postoperative period. The percent rose from 35 to 62 during the 1st to the 3rd week, and varied between 45 and 60% thereafter. The absolute number of T cells rose gradually from 100 cells/mm³ to 350 cells/mm³ during the 1st to 7th weeks. However, the T-cell level at 7 wk was still fivefold decreased as compared to normals (Fig. 2).

Percent and absolute number of B cells in the peripheral blood during the 7-wk postoperative period. Fig. 3 shows that the mean percent of B lymphocytes of all 10

TABLE II

T and B Lymphocytes in Patients with Heart Failure

Patient number	T lymphocytes	Absolute T lymphocytes	B lymphocytes	Absolute B lymphocytes	Monocytes
	%	*counts/mm³*	%	*counts/mm³*	%
1	75	864	16	184	20
2	66	729	20	221	22
3	48	726	9	136	21
4	87	5,043	5	290	13
5	100	2,090	8	167	14
6	82	945	10	115	15
7	65	1,368	33	695	5
Mean	75	1,681	14	258	16

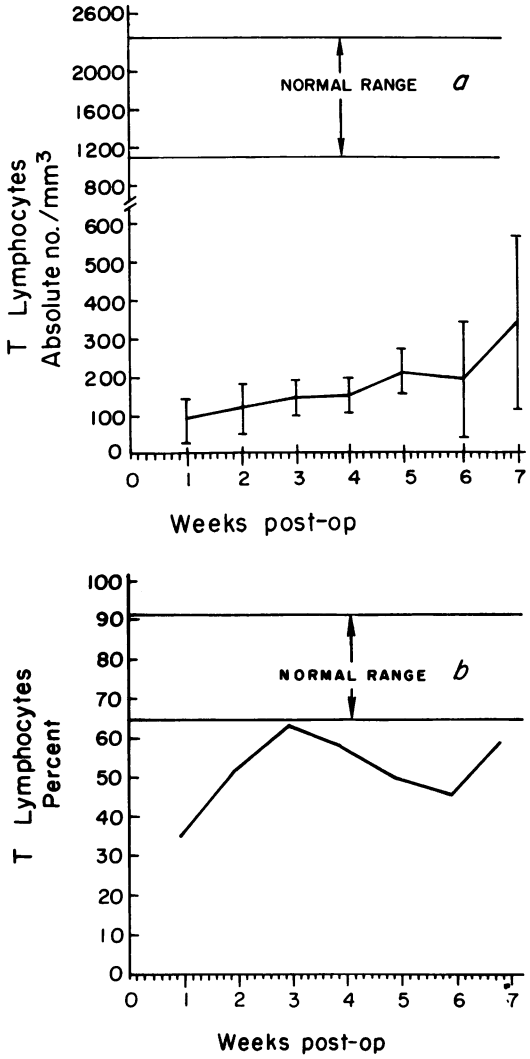

FIGURE 2 (*a*) Mean absolute T-lymphocyte count for each of the 7 wk postoperative in 10 patients. Brackets show the standard error of the mean. (*b*) Mean percent of T lymphocytes for each of the 7 wk postoperative in 10 patients.

patients was elevated during the entire 7-wk period. The highest percent (45–50%) was noted during the first 2-wk. Thereafter, the values remained stable at 35–40%. The absolute B-cell count varied between 100 and 200 cells/mm³ during this time. The 1st- and 7th-wk values showed a two to fourfold decrease as compared to normals.

Percent and absolute number of T and B lymphocytes in patients 3–60 mo postoperative. Table IV shows the percent and absolute T and B lymphocyte counts of patients examined during clinic visits 3–60 mo after cardiac transplantation. All patients were being treated with at least 25 mg of prednisone and 50 mg of azathioprine per day. The mean T-lymphocyte count of 14 determinations carried out in 10 patients was 467 cells/mm³, with a range of 27–1,680 cells/mm³. Seven of the patients showed a severe T lymphopenia (< 500 T cells/mm³). The three longest surviving patients (53–60 mo) showed higher T-cell counts (680–1,680 cells/mm³).

The mean absolute B-lymphocyte count was 95 cells/mm³ with a range of 21–277 cells/mm³. A severe B lymphopenia (< 100 cells/mm³) was noted in five patients. A comparison of the mean T- and B-cell counts of these long-term patients and normals indicates that the absolute levels of both types of lymphocytes are similarly depressed (approximately fourfold). Therefore, the relative specificity of action of the immunosuppressive regimen on T cells noted early after transplantation is no longer present late in the course of maintenance treatment.

The mean percent of T (71%) and B (26%) cells in these patients was similar to that of normals. However,

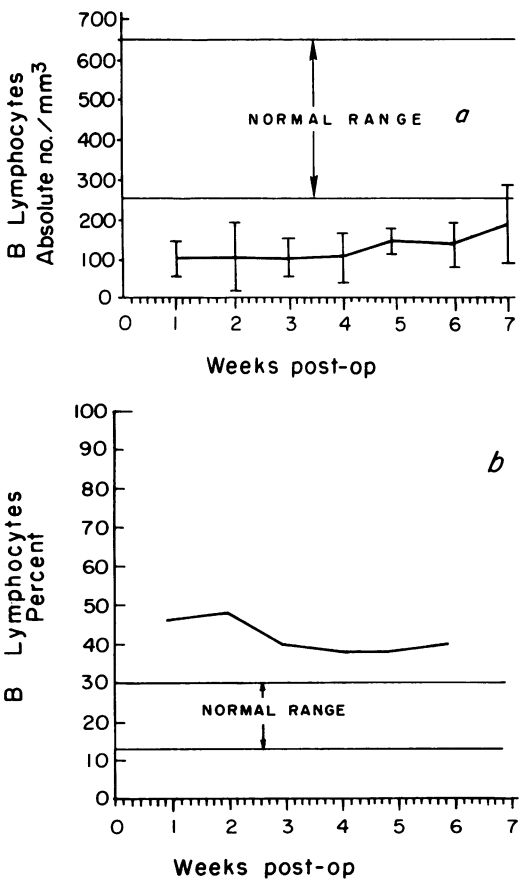

FIGURE 3 (*a*) Mean absolute B-lymphocyte count for each of the 7 wk postoperative in 10 patients. Brackets show the standard error of the mean. (*b*) Mean percent of B lymphocytes for each of the 7 wk postoperative in 10 patients.

TABLE III

Percent of T and B Lymphocytes from Normal Controls before and after Incubation with Serum from Heart Transplant Patients*

Before				After			
Cell donor	Cyto-toxicity	T cells‡	B cells	Serum donor	Cyto-toxicity	T cells	B cells
		%				%	
1	85	88	16	1	93	96	23
				2	83	86	33
2	80	84	13	1	81	85	36
				2	81	85	15

* 1 h at 37°C in undiluted serum; yield of viable cells was >95% as judged by cell count and trypan blue exclusion.
‡ Percent kill adjusted for monocyte contamination.

in individual patients the sum of the percent of T and B cells was considerably lower (67–85%) or higher (129–152%) than normals. This suggests the presence of a "null" population of cells in the former case and persistent Ig coating of T cells in the latter.

Relationship between the percent and absolute count of T lymphocytes and rejection episodes. Fig. 4 shows the time course of immunosuppressive therapy, rejection episodes, and percent and absolute T cells in two patients. These patients are not representative of all 10 cases, since in some instances, rejection episodes were preceded by a marked rise in the percent and absolute number of T cells, as in the second episode illustrated in Fig. 4b. In other instances, a less well-defined rise or no rise at all was noted in the 5-day period preceding a rejection episode (Fig. 4a). Institution of high-dose methylprednisolone (1.0 g daily, intravenously for 2–3 days) during rejection crises was frequently followed by an abrupt decrease in the percent and absolute number of circulating T cells (Figs. 4a and 4b). The continued marked fluctuation in the percent of T cells noted during periods of stable therapy (Fig. 4a) was seen in several patients, and could not be related to other clinical or laboratory parameters.

Although statistically significant correlations were not found between the kinetics of the rises in the level of T cells and rejection episodes, it is noteworthy that patients with more than 400 T cells/mm^3 recorded during the first 3 wk post-transplantation sustained twice as many rejection episodes as those with levels consistently below 400 T cells/mm^3.

DISCUSSION

Alterations in the percent and absolute number of T and B lymphocytes in the peripheral blood were assessed in 10 patients treated with ATG, prednisone, and azathioprine after cardiac transplantation. ATG was given daily for the first 3 days and then every other day for 3 additional days. ATG was subsequently discontinued

TABLE IV

T and B Lymphocytes in Long-Term Heart Transplant Patients

Case no.	Post-op	Prednisone	Imuran	Interval since rejection	T lympho-cytes	T lympho-cytes (Absolute no.)	B lympho-cytes	B lympho-cytes (Absolute no.)	Mono:poly*
	mo	*mg/day*	*mg/day*	*days*	%	*cells/mm^3*	%	*cells/mm^3*	%
1	3	47.5	50	12	50	267	27	144	22 m 14 p
2	3	45	75	15	77	157	16	33	4 p
	4	85	75	Rejecting	50	—	22	—	28 p
3	4	60	100	—	62	402	13	84	11 m 5 p
4	5	75	50	82	93	1,116	3	36	11 p
	10	30	100	234	53	101	30	57	7 m 8 p
	11	30	100	276	97	77	20	16	3 m 19 p
	12	30	100	283	73	223	56	172	2 m 6 p
5	18	32.5	50	331	74	27	78	28	3 m 7 p
6	22	27.5	200	238	48	312	37	240	19 m 27 p
7	23	27.5	100	644	81	211	8	21	31 m 17 p
	24	27.5	100	664	59	281	8	38	40 m 21 p
8	53	70 QOD‡	200	1,182	72	680	15	142	4 m 8 p
9	54	65 QOD‡	200	609	91	1,003	4	44	9 m 4 p
10	60	30	200	229	85	1,680	14	277	24 m 1 p
Mean					71	467	26	95	15 12

* Ficoll purified cells.
‡ Alternate day.

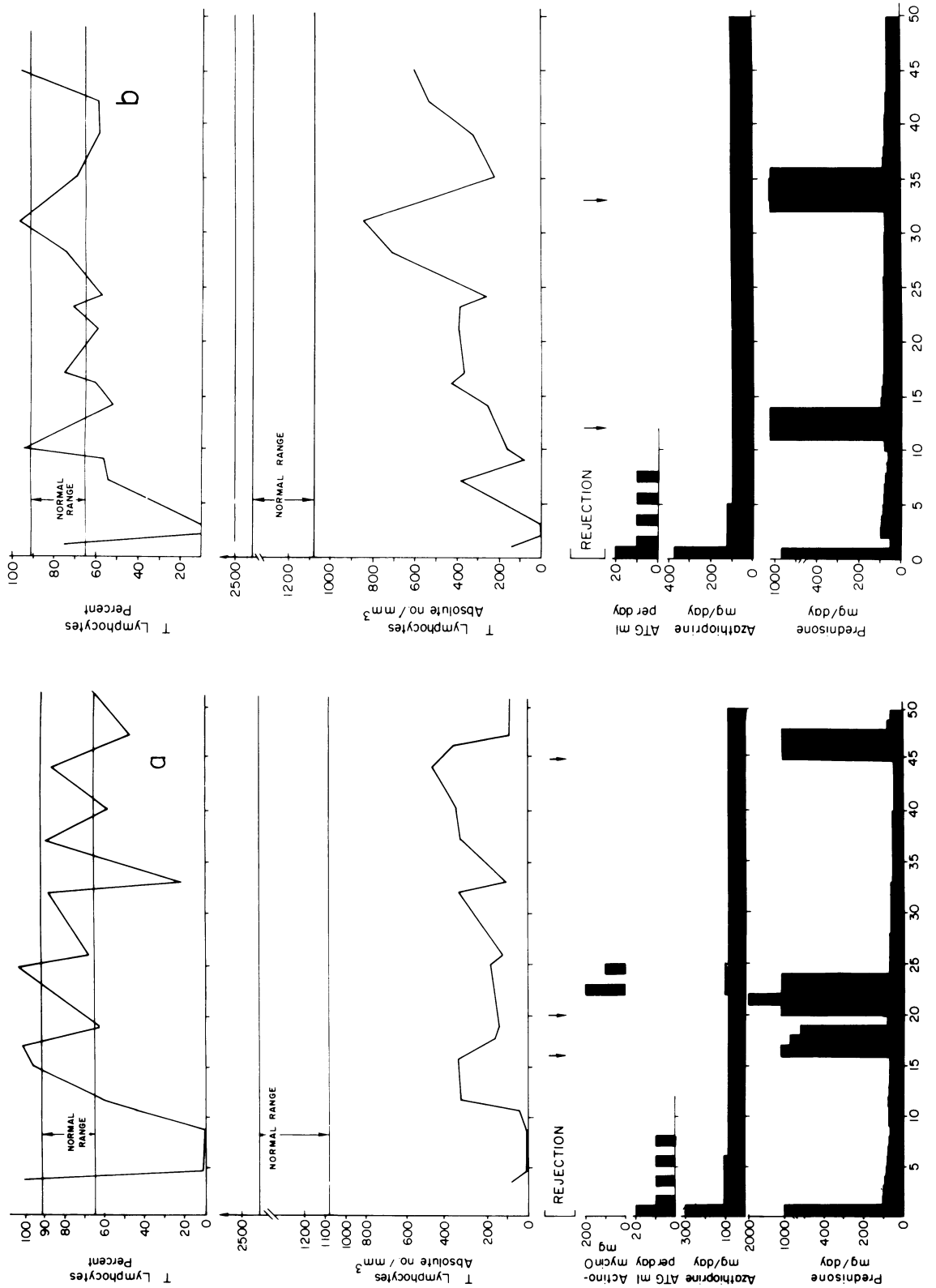

FIGURE 4 (*a* and *b*) Chronological relationship between percent and absolute T-lymphocyte counts and rejection episodes in two patients.

except for occasional use during rejection crises. Treatment with prednisone (or methylprednisolone) and azathioprine was begun on the day of transplantation and continued during the entire postoperative course.

The percent of T cells was determined by an in vitro cytotoxicity assay with a specific goat antihuman T-cell antiserum (13). This assay is preferable to the spontaneous sheep erythrocyte rosette technique in certain disease entities, since alterations in the T-cell surface (such as coating with ATG) can block rosetting and result in spuriously low values for the percent of T cells (16).

Evaluation of control patients with congestive heart failure similar to that presented by our patients preoperatively showed that the mean percent of T cells (75%) and mean absolute T-cell count (1,681/mm^3) were similar to normal (79%, 1,600 T cells/mm^3). However, the mean percent of B cells (14%) and mean absolute B-cell count (258/mm^3) were significantly decreased as compared to normal (20% B, 407 B cells/mm^3).

After cardiac transplantation a characteristic early pattern of change was noted in the levels of T and B cells. During the 1st postoperative wk, the percent of T cells dropped below 10% in almost all cases in association with a concomitant rise in the percent of B cells. Thereafter, the percent of T cells rose abruptly, approaching normal levels. Frequently this rebound was associated with a decrease in the percent of B cells, but in a few cases the percent of B cells remained elevated. In the latter cases the sum of the percent of T and B cells rose well above 100%. This paradoxical finding is most likely explained by coating of T lymphocytes with human immunoglobulin so that they are incorrectly identified as B cells (surface immunoglobulin-bearing cells). Evidence for this point was obtained by demonstrating a rise in the percent of B cells and the sum of the percent of T and B cells in normal lymphocytes after incubation in vitro with serum from patients receiving ATG. Both ATG and antibodies to ATG circulate in the blood of some patients during ATG therapy (17). ATG-coated T cells could therefore bind anti-ATG antibodies to the cell surface and "masquerade" as B cells.

Although the immunosuppressive regimen showed specificity for T-cell depletion, as judged by alterations in the percent of T and B cells, the absolute number of both T and B cells was markedly decreased during the 1st postoperative wk. In almost all cases, the T-cell count was below 200 cells/mm^3, and the B-cell count was similarly depressed. However, the depletion of T cells was considerably more profound than B cells, since the normal absolute T-cell count is four times greater than that of B cells. Similar acute alterations in the percent and absolute numbers of T and B cells in the blood of normal volunteers have been induced by administration of high doses of prednisone alone for short periods of time (18, 19).

However, a recent study of alterations in peripheral blood T cells in patients receiving renal allografts suggests that ATG, prednisone, and azathioprine produce a more profound T-cell lymphopenia than prednisone and azathioprine alone (20). T-cell values may be spuriously low in the latter study, since the sheep erythrocyte rosette technique was used. ATG has been shown to block rosette formation, and coating of the T-cell surface may persist long after ATG has been cleared from the serum.

During the 2nd week after transplantation the percent of T cells rose from 35 to 62%, and varied between 45 and 60% thereafter. The absolute T-cell count rose from 100 to 350 cells/mm^3. The latter figure is still fivefold decreased as compared to that of normals. Although the percent of B cells was elevated (35–50%) during the entire study, the mean absolute B-cell count remained below the range of normals. However, the B-cell level at 50 days postoperative ($\sim$200 cells/mm^3) was not significantly different from that of nonimmunosuppressed patients with cardiac failure (258 cells/mm^3). The selective T-cell depletion noted during the 1st postoperative wk is, therefore, maintained through the 7th postoperative wk.

Long-term follow-up of patients 3–60 mo after transplantation showed that although the mean percent of T (71%) and B (26%) cells had returned to normal, the absolute T-cell (467 cells/mm^3) and B-cell (95 cells/mm^3) counts were considerably decreased. A severe B-cell depletion ($<$ 50 cells/mm^3) was observed in one-half of these patients. This suggests that the target cell specificity of the present long-term immunosuppressive regimens (i.e. azathioprine and prednisone) may not be satisfactory.

The chronological relationship between the percent and absolute count of T cells and rejection crises was variable. In some instances, there was a clear-cut rise in both parameters in the 5-day period preceding a biopsy-proven rejection crisis (Fig. 4). In other cases, rejection episodes were not preceded by rises. Statistical analysis of the time intervals between rejection episodes and peaks in the percent and absolute T-cell count show no significant chronological relationship in the 10 patients analyzed. However, patients with absolute T-cell counts above 400 cells/mm^3 during the first 3 wk postoperative had twice as many rejection episodes as those below this level.

The greater incidence of rejection episodes in patients with high T-cell levels and those instances of rejection crises preceded by rises in the T-cell levels suggest that measurement of T-cell levels in transplant patients may

be a useful adjunctive diagnostic aid in determining adequacy of immunosuppression.

ACKNOWLEDGMENTS

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