De Novo Malignancies after Liver Transplantation Using Tacrolimus-Based Protocols or Cyclosporine-Based Quadruple Immunosuppression with an Interleukin-2 Receptor Antibody or Antithymocyte Globulin

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BACKGROUND. Although conventional immunosuppression after liver transplantation consists of cyclosporine A (CsA), steroids, and azathioprine, recently introduced protocols entail CsA-based quadruple induction protocols or tacrolimus-based combinations. These protocols aim to reduce the rejection rate and the considerable morbidity related to the side effects of additional immunosuppressive treatment, but have not yet been analyzed regarding their long term de novo neoplastic risk.

METHODS. From September 1988 to May 1994, 500 liver transplantations were performed in 458 patients. The median follow-up was 50 months (range, 0.3–97 months) for all patients. Conventional triple therapy was implemented in 25 patients, CsA-based quadruple induction therapy using an antilymphocyte globulin preparation (ATG) in 190 patients, an interleukin-2 receptor antibody (BT563) in 141 patients, and tacrolimus-based dual or triple immunosuppression in 102 patients. The different protocols were evaluated in four randomized and two nonrandomized prospective trials.

RESULTS. De novo neoplasias were detected in 33 patients (7.2%) and were comprised of lymphomas (n = 7), skin malignancies (n = 8 lesions in 7 patients), intraepithelial neoplasias of the cervix uteri (n = 7), breast carcinoma (n = 3), lung carcinoma (n = 3), and other malignancies (n = 6). The incidence of de novo neoplasias did not differ in the different trial arms. Only a positive T-crossmatch and a low $CD4^+/CD8^+$ ratio in patients receiving CsA-based immunosuppression demonstrated a significant correlation with the development of a de novo tumor in a multivariant logistic regression analysis.

CONCLUSIONS. The development of de novo neoplastic diseases after liver transplantation with the use of CsA-based quadruple induction protocols or tacrolimus-based regimens for immunosuppresion was assessed over the long term. Recently introduced immunosuppressive protocols did not alter the posttransplant de novo tumor rate. Patients with a low $CD4^+/CD8^+$ ratio during CsA-based therapy or a positive T-crossmatch were identified to be at an increased risk for the development of a de novo malignancy. ***Cancer* 1997;80:1141–50.**

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The correlation between immunosuppression and malignancy already had been addressed after renal transplantation in the pre-

cyclosporine A (CsA) era and in otherwise immunocompromised patients.[1–4] Posttransplant lymphoproliferative disorders (PTLD) were observed most frequently in combined heart and lung and in liver allograft recipients, correlating to a putative more aggressive immunosuppressive treatment after transplantation of vitally essential organs.[5] Although a relationship between OKT3 treatment, Epstein–Barr virus (EBV) seroconversion, and the development of PTLD was observed among liver transplant recipients, the role of primary immunosuppressive regimens in fostering an environment prone to a malignant transformation remains more elusive.[6,7] An additional risk confered by CsA was suspected due to its inhibitory effects on apoptosis within B cells, but could not be confirmed in recent studies.[8–10]

The authors have reported their encouraging early experience with a quadruple protocol including a monoclonal antibody (BT563) directed against the interleukin 2 (IL-2) receptor (CD25) and with tacrolimus-based regimens.[11,12] To the authors' knowledge, no follow-up data on patients undergoing immunosuppression using an IL-2 receptor antagonist or tacrolimus-based triple therapy have been reported so far. Previous reports on the risk of antilymphocyte globulin quadruple regimens are confined to a retrospective study on PTLD after renal transplantation.[13] Studies of de novo malignancies developing during tacrolimus-based dual therapy did not indicate an elevated risk after renal transplantation, whereas an increased incidence of EBV infection and associated PTLD was observed after pediatric liver transplantation.[14,15] In the current study, the authors report the first follow-up data regarding the development of de novo malignancies after four randomized and two nonrandomized prospective immunosuppressive trials.

MATERIALS AND METHODS

Patient Selection

From September 1988 to May 1994, 500 liver transplantations were performed in 458 patients at the Virchow Medical Center (450 [98.3%] in adults and 8 [1.7%] in children age $<$ 16 years). Anti-EBV immunoglobulin G was detected prior to transplantation in 449 of 458 patients (98.0%). The primary diagnoses in all 458 patients were postnecrotic cirrhosis after hepatitis C (n = 118), hepatitis B (n = 94), alcohol toxic hepatitis (n = 79), autoimmune hepatitis (n = 13), and cryptogenic forms (n = 16) as well as primary biliary cirrhosis (n = 45), primary sclerosing cholangitis (n = 28), Budd–Chiari syndrome (n = 10), hepatocellular carcinoma (n = 8), hilar cholangiocarcinoma (n = 9), and various other indications (n = 38). In 22 patients undergoing transplantation for a posthepatitis B or C cirrhosis, a hepatocellular carcinoma had developed before transplant in the cirrhotic tissue. All patients remained hospitalized for at least 4 weeks after transplantation. Thereafter, patients were checked during close follow-up examinations. Due to the unique geographic situation of former West Berlin, many graft recipients were local residents, allowing for a follow-up of all patients directly in the outpatient department of the study clinic. Along with political changes, potential liver allograft recipients were increasingly allocated from other federal states. However, the monitoring program still has scheduled at least one annual presentation at the Virchow Medical Center for all nonlocal graft recipients during long term follow-up. Moreover, data on these patients, who underwent a check-up at regional institutions in the meantime, were communicated to permit the continuous supervision of care by the transplant team. Three patients (0.7%) were lost to follow-up.

Liver Transplantation

Grafts were preserved almost exclusively using University of Wisconsin solution. Few organs were preserved using Euro Collins (two organs) and Bretschneider's HTK solution (two organs). The surgical procedure was performed in a standardized technique, comprising a venovenous bypass and completion of all four vascular anastomoses prior to reperfusion. In all but 39 cases, which required a biliodigestive anastomosis due to the underlying disease, the biliary reconstruction was performed as a side-to-side choledochocholedochostomy.[16] All patients underwent a concomitant treatment protocol as described previously.[17]

Primary Immunosuppression

A total of 403 patients were enrolled in immunosuppressive trials. The other 55 patients underwent liver transplantation during the early phase of the study program or did not fulfill certain entry criteria (age $>$ 18 years, no retransplantation, no multiorgan transplantation). These 55 patients mainly received the authors' standard quadruple drug induction regimen comprised of CsA, azathioprine, low dose steroids, and an antithymocyte globulin preparation (ATG; Fresenius, Bad Homburg, Germany).

The trials evaluating various primary immunosuppressive protocols and the references for early trial results are depicted in Table 1.[11,12,18] In four of the six prospective trials (Trials II, III, IV, and V), patients were randomly assigned to the different treatment protocols. In Trial I, 33 patients each consecutively had undergone quadruple immunosuppression using ATG or a monoclonal anti-IL-2 receptor antibody (BT563; Biotest GmbH, Dreieich, Germany). Trial VI

TABLE 1
Prospective Immunosuppressive Trials, Assignment of 403 Enrolled Patients, and Numbers of Patients Developing a De Novo Neoplasia

Trial	Design	Ref.	No. of transplants	Patients	De novo tumors[a]	Median observation time (mos) (range)
I	CsA-based quadruple: BT563 vs. ATG; prospective, controlled	11	oLTX 31-106	33 vs. 33	2 vs. 6	78 (0.3–88)
II	Tacrolimus dual vs. CsA-based quadruple (ATG); prospective, randomized, controlled	12	oLTX 107-236	61 vs. 60	5 vs. 6	63 (0.6–73)
III	CsA-based quadruple: BT563 vs. ATG; prospective, randomized, controlled	18	oLTX 237-325	39 vs. 41	2 vs. 3	50 (0.5–57)
IV	Tacrolimus dual vs. tacrolimus triple; prospective, randomized, controlled		oLTX 326-379	21 vs. 21	1 vs. 2	43 (0.4–47)
V	CsA-based quadruple (BT563); prospective, randomized, placebo-controlled		oLTX 384-43	21 vs. 21	—	38 (0.8–41)
VI	CsA-based quadruple (BT563); prospective		oLTX 443-500	52	3	31 (1.0–35)

Ref: reference of early trial results; oLTX: number of liver transplants; CsA: cyclosporine A, ATG: antilymphocyte globulin.
[a] Number of patients developing a de novo neoplasia.

was a one-armed study investigating the currently unapproved antibody BT563.

Briefly, CsA-based protocols were comprised of either conventional triple therapy, of the authors' standard quadruple drug induction regimen entailing ATG, or of a another sequential quadruple drug protocol using BT563.[11,19] Except for the ATG or BT563 treatment, an almost identical immunosuppressive regimen was applied irrespective of the primary protocol group. CsA was started after surgery as a parenteral dose of 1–2 mg/kg body weight twice a day and was switched to an oral intake of 5 mg/kg body weight twice a day on posttransplant Day 5. Subsequent dosing was adjusted according to whole blood levels with the goal of 600 to 900 ng/mL as measured by a polyclonal fluorescence polarization immunoassay (TDX Assay, Abbott Laboratories, Abbott Park, IL). Methylprednisolone was given prior to reperfusion and directly after transplantation at a dose of 500 mg intravenously (i.v.) each. Prednisolone was begun as a single oral dose of 1 mg/kg body weight that was tapered to 20 mg/day during the first month. Azathioprine was started as parenteral administration of 25 mg/day until 1 week after transplantation; on posttransplant Day 7 the dosage was increased to 1–2 mg/kg body weight orally. Intake was reduced or interrupted according to peripheral leukocyte counts. ATG was given in a parenteral dose of 5 mg/kg body weight/day for 7 days. BT563 was administered i.v. for 12 days at a daily dose of 10 mg. In the first tacrolimus dual protocol (Table 1; Trial II), as part of a European multicenter trial the drug was administered as a single i.v. dose of 0.075 mg/kg body weight within 8 hours after surgery and 2 parenteral doses of 0.075 mg/kg body weight over a period of 4 hours each on posttransplant Days 1 and 2. Five months after starting the trial, an amendment to the initial administration of tacrolimus was made and the i.v. dose was reduced to 0.03 to 0.05 mg/kg body weight. Oral intake was started 3 days posttransplant (2×0.15 mg/kg/day prior to the amendment, and 2×0.10 mg/kg/day afterward). Methylprednisolone was administered twice at an i.v. dosage of 500 mg prior to reperfusion and immediately after transplantation. Prednisolone was given once daily tapered from 20 mg to 15 mg orally as a maintenance dosage. In the dual tacrolimus protocol in Trial IV (Table 1), oral intake (2×0.05 mg/kg/day) was administered during the immediate postoperative course. Tacrolimus-based triple therapy (Table 1; Trial IV) used an initial oral dosing of 2×0.03 mg/kg/day as well as oral azathioprine (2×1–2 mg/kg/day) and prednisolone.

Rejection Episodes

Rejection episodes were suspected in case of scant production of light bile or biochemical graft dysfunction as defined by rising serum levels of bilirubin or hepatic enzymes ($> 50\%$ above initial values) without evidence of mechanical causes or infection and were confirmed by biopsy.[20] Core needle liver biopsies also were routinely obtained on posttransplant Day 7.

Doppler ultrasound was performed if indicated to rule out hepatic artery or portal vein thrombosis. If suspicion of a vascular complication prevailed, diagnosis had to be confirmed by angiography. Bile leakage or biliary obstruction were excluded by cholangiography. A diagnosis of chronic or ductopenic rejection relied largely on the evidence of cholestasis with an interlobular and septal duct loss as well as on obliterative arteriolar lesions, portal tract fibrosis with linkage between central veins and portal triads, and on the absence of findings concordant with viral hepatitis.

Treatment of Rejection

Initial therapy of cellular rejection episodes was comprised of a 3-day course of high dose steroids (i.e., 500 mg/day of i.v. methylprednisolone). Steroid-resistant episodes were treated with OKT3 (5 mg/day) over 5–7 days. Tacrolimus became available to the study center in May 1990 and was applied as rescue agent in OKT3 nonresponders, or if changes indicating a chronic rejection became evident. After gaining more experience with tacrolimus, direct conversions for steroid-resistant cellular rejections and uncomplicated cellular rejections were implemented. A conversion began with continuation of oral steroids and administration of oral tacrolimus. Initial dosing ranged from 0.07–0.1 mg/kg body weight twice a day. Further adjustments were related to toxicity and response or graft function.

Lymphocyte Profile Determinations

Peripheral blood mononuclear cells for determination of lymphocyte surface profiles according to the National Committee for Clinical Laboratory Standards protocol were obtained from ethylenediamine tetraacetic acid (EDTA) anticoagulated blood 18–48 months (34 ± 27 months) posttransplant. Using ammonium-chloride-EDTA-buffer red cell lysis (Ortho; Raritan, NJ), the samples were stained with monoclonal antibodies (MoAbs) (all MoAbs from Coulter-Immunotech, Marseille, France), centrifuged, and resuspended in phosphate-buffered saline. Expression of surface markers was quantified flow cytometrically using a FACScan (Becton Dickinson, Sunnyvale, CA) operating the LYSIS II software (Becton Dickinson) as previously described.[21] A minimum of 5000 gated lymphocytes were acquired, attaining an estimated precision at a coefficient of variation approximating 15% at the 1% level.[22] Absolute counts of lymphocyte subpopulations were calculated according to the lymphocyte count/nL as determined by a hematologic counter (NE-7000; Sysmex, Kobe, Japan).

Statistical Evaluation

Comparison of groups with regard to patient and graft survival were performed with Kaplan–Meier estimates and the log rank test. Data were expressed as mean ± standard error of the mean. Comparisons between groups were made by the Wilcoxon rank sum test for continuous variables and by the chi-square test for categoric variables. A multivariant logistic regression analysis was performed to adjust risk ratios for potential confounding covariates. Variables demonstrating a significant correlation in a univariate analysis or refering to the treatment of the patients (mainly primary and additional immunosuppression) were included.

RESULTS

De novo malignant disorders were observed in 33 of 458 patients (7.2%) after liver transplantation. One patient had more than one tumor. At the time of transplantation, the age of the patients later developing a de novo neoplasia was 46 ± 14 years compared with 45 ± 18 years in the no tumor group. Of the 458 patients, 267 were male and 191 were female. Female patients were at a higher although not significantly different risk of having a de novo tumor than male patients (n = 17 [8.9%] vs. n = 16 [6.0%]; the difference was not significant). The primary diagnoses in these 33 patients were postnecrotic cirrhosis due to hepatitis B (n = 10), hepatitis C (n = 7), alcohol toxic hepatitis (n = 5), cryptogenic forms (n = 3), primary biliary cirrhosis (n = 3), primary sclerosing cholangitis (n = 2), and other indications (n = 3) and did not differ significantly from the total series. The median observation time after transplantation was 50 months (range, 0.3–97 months) for all patients; the median time elapsing after transplant until the diagnosis of the de novo malignant disease was 43 months (range, 1–67 months). The cumulative incidences of de novo neoplasia 1, 3, and 5 years after transplant were 1.7%, 5.9%, and 14.6%, respectively. The various tumors, the patients' gender and age, as well as the primary immunosuppression and treatment for rejection are depicted in Table 2. De novo neoplastic lesions of the lymphoreticular system, the skin, and the cervix uteri affected a total of 7 patients (21.2%). A heterogenous group of other malignancies was comprised of female breast carcinoma (n = 3), lung carcinoma (n = 3), carcinoma of the tongue (n = 2), gastric carcinoma (n = 1), embryonal testicular carcinoma (n = 1), papillary thyroid carcinoma (n = 1), and Kaposi's sarcoma (n = 1). Patient survival was assessed 6 years after transplant and did not differ between those developing a de novo malignancy and all other patients (Fig. 1).

TABLE 2
De Novo Tumors and Characteristics of the 33 Patients

De novo tumor	Primary diagnosis	Gender	Age (yrs)	Months after transplant to diagnosis of the de novo tumor	Primary immunosuppression	Rejection treatment
Lymphoma						
Hodgkin's disease	Protoporphyria	Male	22	67	Cyclosporine (ATG)	
Hodgkin's disease	PSC	Male	51	28	Cyclosporine (ATG)	
Non-Hodgkin's lymphoma	Hepatitis C	Female	59	59	Cyclosporine (ATG)	OKT3[a]
Non-Hodgkin's lymphoma	Hepatitis B	Male	55	49	Cyclosporine (ATG)	
Non-Hodgkin's lymphoma	Hepatitis C	Female	49	13	Cyclosporine (ATG)	OKT3,[a] tacrolimus rescue
Non-Hodgkin's lymphoma	Hepatitis B	Male	60	6	Cyclosporine (BT563)	
EVB-associated PTLD	Crigler-Najjar syndrome	Male	17	5	Cyclosporine (BT563)	Steroids, tacrolimus rescue
Skin tumors						
Squamous cell carcinoma	Hepatitis B	Male	55	63	Cyclosporine (ATG)	OKT3[a]
Squamous cell carcinoma	Hepatitis C	Male	56	36	Cyclosporine (ATG)	
Squamous/basal cell carcinoma	Hepatitis C	Male	63	23/30	Cyclosporine (triple)	
Basal cell carcinoma	Hepatitis C	Female	63	59	Cyclosporine (ATG)	OKT3[a]
Basal cell carcinoma	Alcohol toxic	Male	51	55	Cyclosporine (ATG)	
Spinalioma	Hepatitis B	Male	57	7	Cyclosporine (ATG)	
Merkel cell tumor	Autoimmune	Female	50	21	Cyclosporine (BT563)	Steroids
Cervical intraepithelial neoplasia						
CIN I	Hepatitis C	Female	39	9	Tacrolimus (dual)	
CIN II	Hepatitis B	Female	27	27	Tacrolimus (dual)	
CIN II	Cryptogenic	Female	27	9	Tacrolimus (triple)	
CIN III	Hepatitis B	Female	31	59	Cyclosporine (ATG)	
CIN III	Hepatitis B	Female	27	26	Cyclosporine (BT563)	
CIN III	Alcohol toxic	Female	29	12	Cyclosporine (ATG)	
CIN III	Cryptogenic	Female	37	1	Cyclosporine (ATG)	OKT3[a]
Others						
Breast carcinoma	Alcohol toxic	Female	43	35	Tacrolimus (dual)	Steroids
Breast carcinoma	PBC	Female	64	16	Tacrolimus (dual)	
Breast carcinoma	Hepatitis C	Female	47	3	Cyclosporine (BT563)	Steroids
Lung carcinoma	Hepatitis B	Female	61	68	Tacrolimus (dual)	Steroids
Lung carcinoma	Alcohol toxic	Male	56	52	Cyclosporine (BT563)	Steroids
Lung carcinoma	PBC	Male	58	30	Cyclosporine (ATG)	
Tongue carcinoma	Hepatitis B	Female	58	38	Cyclosporine (ATG)	
Tongue carcinoma	Alcohol toxic	Male	49	17	Tacrolimus (triple)	Steroids
Gastric carcinoma	Cryptogenic	Male	57	86	Cyclosporine (ATG)	
Embryonal testicular carcinoma	PSC	Male	38	47	Cyclosporine (ATG)	Steroids
Papillary thyroid carcinoma	PBC	Female	25	35	Tacrolimus (dual)	
Kaposi's sarcoma	Hepatitis B	Male	43	13	Cyclosporine (BT563)	

PBC: primary biliary cirrhosis; PSC: primary sclerosing cholangitis; autoimmune: postautoimmune hepatitic cirrhosis; EBV: Epstein–Barr virus; PTLD: posttransplant lymphoproliferative disorder; CIN: cervical intraepithelial neoplasia; ATG: antilymphocyte globulin.

[a] OKT3 treatment was always preceded by at least 1 3-day course of steroids.

Lymphoma

In the group of patients with lymphoproliferative disorders (seven patients), two patients had Stage IIB (Cotswald staging classification) Hodgkin's disease and at last follow-up were free from recurrence 26 and 57 months, respectively, after completion of chemotherapy (doxorubicin, bleomycin, vinblastine, and dacarbazine/cyclophosphamide, vincristine, procarbazine, and prednisone). In one patient developing a non-Hodgkin's lymphoma, complete remission was achieved after chemotherapy. Non-Hodgkin's lymphomas were fatal shortly after diagnosis and prior to the onset of therapy in two patients; in one patient the diagnosis was made incidentally after death from a cytomegalovirus pneumonia. The only EBV-associated PTLD occurred in a 17-year-old male patient with a posttransplant EBV seroconversion that was confined to the tonsils and could be treated successfully by a reduction of immunosuppression. The time elapsing from trans-

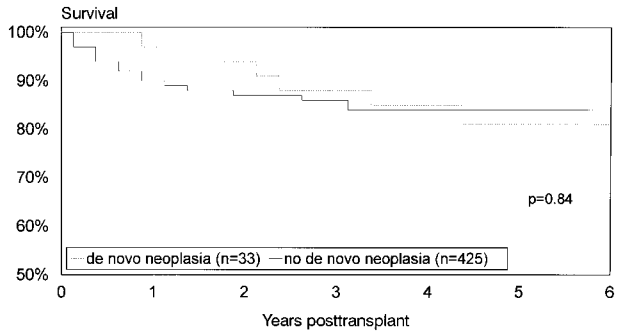

FIGURE 1. Survival rates after liver transplantation with regard to the development of a de novo neoplasia.

plantation to the diagnosis of a de novo lymphoma in 2 patients undergoing cyclosporine-based quadruple induction therapy using the IL-2 receptor antibody BT563 was 5 and 6 months, respectively. This period was significantly shorter ($P = 0.05$) than in the other five patients who had undergone ATG induction therapy (Table 2).

Skin Tumors

Skin tumors (eight tumors in seven patients) were comprised of squamous cell (three tumors) and basal cell carcinoma (three tumors) in five patients as well as a spinalioma (one tumor) and a Merkel cell carcinoma (one tumor). Surgical resection was performed in all seven patients and was curative in all but the one patient with the Merkel cell carcinoma. This patient, a 52-year-old female, underwent 7 cycles of a combined radiotherapy/chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) and died 19 months after diagnosis of the tumor.

Cervical Intraepithelial Neoplasia/Female Breast Carcinoma

Lesions of the cervix uteri were comprised of three degrees of cervical intraepithelial neoplasia (CIN): CIN-1 (one patient), CIN-2 (two patients), and CIN-3 or carcinoma in situ (four patients). Recurrent neoplastic disease could not be detected in any patient after either conization (two patients) or hysterectomy (five patients). However, one patient died of a recurrence of the primary disease, an alcohol toxic cirrhosis. Breast carcinoma accounted for three de novo malignancies. No distant metastases were found, and the estrogen receptor status was positive. Treatment was comprised of a lumpectomy (two patients; T2N0, T1N1) and mastectomy (one patient; T4N0); all three patients received adjuvant radiotherapy/antihormonal therapy. At last follow-up, the patients were free from recurrent disease 8, 26, and 45 months after resection, respectively. In the group of female neoplasias (n = 10) (i.e., CIN and breast carcinoma) 5 lesions each were observed during tacrolimus-based (4.9%) or CsA-based primary immunosuppression (1.4%), which rendered female patients receiving tacrolimus at a higher although not significantly increased risk of developing either a CIN or breast carcinoma (Table 2).

Other Tumors

Lung carcinoma was observed in three patients and was comprised of adenocarcinomas (two patients; both stage IIIB) and a squamous cell carcinoma (one patient; Stage IIIA). Combined palliative radiotherapy/chemotherapy (two patients) or neoadjuvant radiotherapy and resection (one patient) were performed. Two patients died 11 and 13 months, respectively, after diagnosis; at last follow-up, one was alive with a recurrent malignancy 4 months after surgery. Both tongue malignancies, a T1N0 and a T3N0 lesion, were surgically resected. A lymphonodular recurrence was detected in the patient with the T3N0 lesion 20 months after resection; after a surgical reintervention, it was treated by radiotherapy. One patient each with lung carcinoma or tongue carcinoma had undergone a transplant for an alcohol toxic cirrhosis (Table 2). After transplant, at last follow-up, none of these patients had started drinking again. These patients confirmed a former history of cigarette smoking, and one patient with a tongue malignancy was still smoking cigars after the transplant. From the remaining tumor types, a diffuse, infiltrating gastric carcinoma with peritoneal carcinomatosis conferred the poorest prognosis; the patient was still alive 6 months after a palliative gastrectomy. Patients with an embryonal testicular carcinoma (Lugano Stage IIIB), a papillary thyroid carcinoma (T4N1b), and a localized cutaneous Kaposi's sarcoma of the lower leg (Mitsuyasu Stage I) were cured by PEI (cisplatin, etoposide, ifosfamide) chemotherapy, combined thyroidectomy/cervical lymphadenectomy/thymectomy/^{131}iodine therapy, or radiotherapy, respectively. At last follow-up, these patients were alive without malignancy 36, 25, and 66 months after diagnosis, respectively.

Primary Immunosuppression

The incidence of de novo malignancy according to the primary immunosuppressive treatment was 8.9% (ATG; 17 of 190 patients) and 4.9% (BT563; 7 of 141 patients) in the groups receiving CsA-based quadruple regimens, 7.8% (8 of 102 patients) in the group undergoing tacrolimus-based immunosuppression, and 4.0% (1 of 25 patients) in patients receiving conven-

TABLE 3
Analysis of Parameters Related to the Host Immunologic State with Regard to the Occurrence of a De Novo Tumor

	De novo tumor	No de novo tumor	*P* value	Risk ratio
Cellular rejection	11 (33.3%)	196 (46.1%)	0.09	0.54 (0.26–1.21)
Chronic rejection	2 (6.1%)	17 (4.0%)	0.91	1.34 (0.30–6.02)
OKT3-rejection treatment	5 (15.2%)	42 (9.9%)	0.61	1.15 (0.39–3.39)
Tacrolimus-rejection treatment	2 (6.1%)	50 (11.8%)	0.48	0.93 (0.21–4.12)
Splenectomy/arterial banding	6 (18.2%)	89 (20.9%)	0.92	0.96 (0.51–2.13)
Primary immunosuppression:				
CsA-based	25	331		
Tacrolimus-based	8	94	0.95	1.04 (0.46–2.37)
CMV infection	5 (15.2%)	69 (16.2%)	1.0	0.95 (0.47–2.20)
$CD4^+/CD8^+$ ratio < 0.8 (n = 403)	17 (51.5%)	92 (24.9%)	0.001	3.35 (2.48–4.84)
Relative $CD4^+$ count < 30% (n = 403)	16 (48.5%)	116 (31.3%)	0.04	2.17 (1.18–4.53)
T-crossmatch (n = 333)	6 (19.4%)	18 (6.0%)	0.02	3.37 (1.44–7.42)
B-crossmatch (n = 333)	4 (12.9%)	22 (7.3%)	0.23	1.52 (0.49–4.69)

95% CI: 95% confidence interval; CsA: cyclosporine A, CMV: cytomegalovirus.

tional triple therapy. These figures showed no significant difference.

The distribution of 30 patients with a de novo tumor who were enrolled in prospective trials evaluating primary immunosuppressive protocols is shown in Table 1 according to the different treatment arms. An even ratio for the incidence of de novo malignancies became evident in all trials except for Trial I, which compared quadruple immunosuppression with ATG and BT563, in which six and two de novo tumors were observed, respectively. This difference did not reach statistical significance. Moreover, three of the six patients undergoing ATG induction therapy had received OKT3 as therapy for steroid-resistant rejection (Table 2).

Immunologic Host-Related Factors

In a univariate analysis, patients with decreased relative counts of $CD4^+$ lymphocytes (<30%), a decreased $CD4^+/CD8^+$ lymphocyte ratio (<0.8) or a positive T-crossmatch were at a significantly higher risk to develop a de novo neoplasia (Table 3). Patients undergoing a cellular rejection tended to have a lower incidence of de novo malignancies. These variables as well as the incidences of chronic rejection and CMV infection, a history of a splenectomy or a banding of the splenic artery, primary and additional immunosuppressive treatment was tested in a multivariant logistic regression analysis. A significantly increased risk of having a de novo malignancy was calculated only for patients with a positive T-crossmatch (risk ratio 1.82; 95% CI, 1.16–3.33; $P = 0.02$) or a $CD4^+/CD8^+$ lymphocyte ratio < 0.8 (risk ratio 1.77, 95% CI, 1.19–2.85; $P = 0.01$). Testing for an interaction with the main primary immunosuppressive drug revealed a significant correlation with a low $CD4^+/CD8^+$ lymphocyte ratio only in patients receiving CsA-based immunosuppression (risk ratio 2.31, 95% CI, 1.26–4.45; $P = 0.004$).

Pretransplant Child-Pugh stage ($P = 0.47$), donor-recipient gender match ($P = 0.21$), and number of histocompatibility antigen matches ($P = 0.87$) were not significantly correlated with the development of a de novo tumor (data not shown).

Lymphocyte Phenotyping

The cell surface profiles of peripheral blood lymphocytes (PBL) obtained after a mean of 34 months posttransplant demonstrated a significant decrease in the $CD4^+/CD8^+$ lymphocyte ratio as well as of the relative percentage of $CD4^+$ lymphocytes when comparing patients in the CsA groups who developed a de novo tumor with those who did not (Table 4). The total $CD3/38^+$ PBL count was significantly elevated in those with a tumor whereas its relative percentage was not affected significantly. Moreover, the total lymphocyte, $CD8^+$ and natural killer (NK) ($CD16/56^+$) cell counts were significantly increased in the de novo tumor group receiving CsA-based primary immunosuppression. No significant differences could be disclosed in the tacrolimus group, in which an elevated $CD19^+$ lymphocyte count in patients without a malignant transformation represented the only finding bearing a tendency.

DISCUSSION

Similar rates of de novo neoplasia were observed in the arms of four randomized and two nonrandomized

TABLE 4
Lymphocyte Surface Profile Determinations According to the Main Primary Immunosuppressive Drug and the Occurrence of a De Novo Tumor

	De novo tumor Cyclosporine A group	No de novo tumor Cyclosporine A group	*P* value	De novo tumor Tacrolimus group	No de novo tumor Tacrolimus group	*P* value
$CD19^+$	130 ± 146	103 ± 105	0.47	103 ± 86	191 ± 155	0.10
$CD19^+$ (%)	8.4 ± 5.1%	8.2 ± 5.7%	0.55	10.8 ± 13.0%	12.0 ± 6.7%	0.13
$CD3^+$	1195 ± 729	990 ± 675	0.13	930 ± 569	1149 ± 710	0.53
$CD3^+$ (%)	68.7 ± 20.0%	80.4 ± 74.7%	0.08	72.6 ± 19.9%	80.4 ± 99.2%	0.36
$CD4^+$	454 ± 221	468 ± 248	0.94	409 ± 292	554 ± 284	0.14
$CD4^+$ (%)	28.7 ± 12.6%	38.6 ± 12.2%	0.0007	33.9 ± 16.0%	36.0 ± 12.0%	0.80
$CD8^+$	694 ± 619	481 ± 464	0.04	486 ± 411	570 ± 537	0.48
$CD8^+$ (%)	36.8 ± 16.1 %	34.2 ± 14.5%	0.33	36.3 ± 20.4%	32.7 ± 13.4%	0.62
$CD8/38^+$	194 ± 143	120 ± 185	0.003	261 ± 338	196 ± 283	0.77
$CD8/38^+$ (%)	10.8 ± 7.0%	9.1 ± 9.3%	0.09	18.1 ± 18.9%	11.5 ± 11.7%	0.33
$CD4^+/CD8^+$	0.83 ± 0.54	1.40 ± 0.83	0.0007	1.40 ± 1.15	1.44 ± 1.09	0.88
Lymphocytes	1694 ± 970	1294 ± 809	0.02	1206 ± 632	1646 ± 940	0.26
$CD16/56^+$	230 ± 236	147 ± 217	0.04	126 ± 108	214 ± 303	0.51
$CD16/56^+$ (%)	12.4 ± 9.7%	11.1 ± 12.7%	0.20	11.3 ± 7.7%	11.9 ± 11.8%	0.77
HLA+	124 ± 208	63 ± 112	0.26	109 ± 171	84 ± 135	0.89
HLA+ (%)	5.5 ± 6.9%	4.6 ± 5.6%	0.76	6.6 ± 10.7%	4.7 ± 5.8%	0.72

counts/μl; (%): relative counts

prospective immunosuppressive trials evaluating conventional triple therapy, CsA-based quadruple protocols, and tacrolimus-based regimens during long term follow-up. To the authors' knowledge, these are the first reported figures for CsA-based quadruple immunosuppression using an IL-2 receptor antibody (BT563) or tacrolimus-based triple therapy. The risk of developing de novo tumors during CsA-based induction therapy with ATG has not been reported previously after liver transplantation. The rate of de novo tumors was not altered by these recently introduced immunosuppressive protocols, which indicates that lower single drug doses in the early posttransplant phase of sequential induction regimens did not impact favorably on the incidence of de novo neoplasia over the long term. In addition, a reported reduction of early rejection rates by CsA-based quadruple protocols or tacrolimus-based immunosuppression[11,12,18] had no untoward effects on posttransplant de novo tumorigenesis.

The overall incidence of 7% after a median follow-up of 50 months corresponds to the increased de novo tumor rates after renal transplantation.[9,10,23] After renal transplantation, various primary immunosuppressive regimens could not be associated consistently with different risks of malignancy as well.[10] Variable CsA dosing and doubts cast on the validity of multicentric databases made the interpretation difficult. However, CsA-based triple protocols using doses lower than those used during its introduction into solid organ transplantation resulted in significantly lower rates of non-Hodgkin's lymphoma and skin tumors.[9] Therefore, it was interesting to note that lymphomas and skin malignancies arose in the CsA-based arms of the authors' trials exclusively. The number of patients undergoing conventional triple therapy only (n = 25) may have been too small to allow for a reliable estimation of the risk of developing a de novo lymphoma or skin carcinoma. With the exception of one patient, all patients with these tumors had received either ATG or BT563 as induction therapy. Moreover, a promoting effect mediated by antibodies blocking lymphocyte functions is emphasized by significantly decreased $CD4^+$ T-cell-counts in patients with de novo tumors in the CsA-based trial arms as well as by the absence of lymphoproliferative disorders in those receiving tacrolimus-baseline immunosuppression. The latter finding contrasts with a study indicating an increased incidence of PTLD along with EBV infection in pediatric graft recipients receiving tacrolimus.[15] In this study as well, almost all children had undergone additional antilymphocytic therapy although mainly for rejection. Only 8 patients in the current trials were children and only 9 of the 458 patients presented with a pretransplantation seronegative EBV status, which has emerged as primary risk factor for PTLD in adults.[7] This difference also may account for normal $CD19^+$ lymphocyte counts in all groups in the current trials, whereas increased posttransplant levels were previously reported to be associated with EBV infection and concomitant PTLD.[24]

Although the overall rate of de novo tumors in the

group of patients receiving primary tacrolimus-based immunosuppression remained within the scope of the figures in the CsA-based trial arms, tumors in female patients, either as de novo breast carcinoma or CIN, were observed at a higher rate.

Experimental data showed that female breast carcinoma may be promoted by an immunologic response; therefore, a reduction of $CD4^+$ T cells and a consecutive decrease in the $CD4^+/CD8^+$ ratio inhibited tumor promotion in mice.[25,26] Moreover, the incidence of de novo breast carcinoma was shown to be lower in chronically immunosuppressed women than the numbers expected for the general female population.[27] In the CsA-based arms of the current trials, both the relative $CD4^+$ T cell count as well as the $CD4^+/CD8^+$ ratio were highly significantly decreased in those patients developing a de novo tumor compared with those who did not. On the contrary, T cell counts neither were reduced nor influenced by the development of a de novo malignancy in the tacrolimus-based treatment arms.

De novo CIN contributed to as many as 7 of the 33 tumors. It was previously suggested that, in view of its already high incidence in the general population and its association with human papillomavirus, CIN probably was underestimated in early reports on tumorigenesis after renal transplantation and may serve as a control for quality of follow-up and screening.[5] The possibility of a reliable prevention resulted in the detection of all lesions as CIN without progression to invasive carcinoma, as well as in their curative resection.

The use of tacrolimus in the treatment of graft rejection has been shown to be a safe and reliable treatment option.[28] Only 2 of 52 patients developed a de novo malignancy after conversion to tacrolimus. Moreover, additional risk factors were evident in both patients (a posttransplant EBV seroconversion and a prior OKT3 treatment, respectively). This observation is in accordance with increased rates of lethal infections in patients after subsequent OKT3 and tacrolimus applications when compared with an early tacrolimus rescue therapy only.[28,29]

The use of OKT3 (n = 47 patients; 13.2%) correlated with the development of a de novo tumor in 5 patients, all were immunosuppressed with CsA and ATG for induction therapy. Three of these patients were enrolled in Trial I, which may account for the slight preponderance of de novo tumors in the ATG group when compared with the BT563 group (six vs. two patients). These findings corroborate with a previous study on lymphoproliferative disorders in renal transplant patients, which revealed an additional risk of quadruple immunosuppression with antilymphocyte preparations exclusively in those undergoing additional OKT3 treatment.[13] The influence of OKT3 on the incidence of de novo malignancies may have been underestimated in the current study, because the overall rate of OKT3 administrations as treatment of steroid-resistant rejections was low.

A significantly higher rate of positive cytotoxic crossmatches in patients developing a posttransplant de novo malignancy is an unprecedented finding. In contrast, a recent study did not indicate a discernible effect of a positive crossmatch on graft survival.[30] The follow-up periods in this report and in the current study were comparable and may have been too short to allow for a significant impact of de novo neoplasias on survival at this time. Because the additional immunosuppressive treatment did not differ between the de novo tumor group and the remaining series in the current study, there is no conclusive explanation for the observed impact of a positive cytotoxic crossmatch. However, activated T-lymphocyte ($CD8/38^+$) and NK cell ($CD16/56^+$) counts were elevated in the de novo tumor group, especially when CsA was used as the primary immunosuppressant. It remains uncertain whether the activation is solely in response to tumor growth or is the expression of a chronic antigenic stimulus, possibly mediated by a positive crossmatch, as well.

The risk of developing a posttransplant de novo malignancy was evaluated for a conventional triple protocol, CsA-based quadruple immunosuppression using ATG or BT563, and during tacrolimus-based dual or triple therapy tumor in six prospective trials. The distribution of de novo tumors was even in the different arms. Few de novo tumors may have evaded diagnosis when patients died from other complications in the early posttransplant period. De novo tumors that could be detected properly did not yet have a substantial influence over survival rates. Outcome was poor in patients with non-Hodgkin's lymphoma and lung carcinoma, whereas skin carcinomas and CINs (i.e., lesions that could be reliably treated after effective screening) had a considerably better prognosis. The consistency of the finding that CsA-based and tacrolimus-based immunosuppressive protocols bore different risks regarding the type of de novo malignancy still need to be substantiated. Although conversion to tacrolimus as treatment of graft rejection emerged as a safe option, the impact of OKT3 applications on de novo tumorigenesis may have been underscored in the current study as the overall treatment rate was low. The previous finding of an increased risk in combination with antilymphocyte preparations was corroborated. It is not clear whether an even more stringent posttransplant screening would result in im-

proved survival figures. However, patients with a positive crossmatch or a low $CD4^+/CD8^+$ ratio during CsA-based therapy are at a higher risk and require particular attention.

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