Immunobiol., vol. 184, pp. 321–335 (1992)

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Autotransplanted Jejunum in Patients with Carcinomas of the Head and Neck: Transport of Immunosurveillance against Tumor Cells?

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Received December 3, 1990 · Accepted in revised form November 4, 1991

Abstract

Autologous jejunum, transplanted as a functional replacement immediately after radical dissection of advanced stages of squamous cell carcinomas of the head and neck and subsequently irradiated, was examined by immunohistochemistry (APAAP/PAP-technique). Biopsies from 9 patients were taken at the time of transplantation and up to 24 months thereafter (group 1) and from 5 patients only once after transplantation (group 2). Twenty-six monoclonal antibodies (mAbs) were used as surface markers to give an overview about phenotypical changes with respect to T-, B- and MΦ-antigens. 1) B cells: a general increase of $CR2^+$ (CD21, $p < 0.01$) could be noticed after transplantation, immunoglobulin positive cells remained unchanged expect for a significant decrease of IgM^+ ($p < 0.01$) and $IgA1^+$ ($p < 0.01$) cells. 2) The number of T cells (CD3+) showed no significant differences although TcR gamma/delta+ cells decreased ($p < 0.01$) in the autotransplant. ICAM-1 (CD54) and IL-2R (CD25) were found on a significant ($p < 0.01$) higher number of cells after transplantation. 3) Cells with M/MΦ morphology showed increased expression of the Fc gamma receptors (CD64, $p < 0.001$; CD32, n.s.; CD16, $p < 0.001$), of the complement receptors CR1 (CD35, $p < 0.001$) and CR3 (CD11b, $p < 0.02$), of HLA-DQ ($p < 0.01$), and of the antigens 25F9 (mature MΦ; $p < 0.01$) and CD4 ($p < 0.02$). Correlation analyses of data obtained from the biopsies of the 14 autotransplanted jejunum cases revealed a $CD35^+$ and a $25F9^+$ subpopulation of M/MΦ. Our findings indicate that despite irradiation autotransplanted jejunum contained cells with immunological capacities. Therefore, the replacement of larynx by autologous jejunum may facilitate not only mechanical but also immunological functions.

Introduction

Squamous cell carcinomas of the upper aerodigestive tract, in particular at advanced stages of disease, represent a severe therapeutical problem since

Supported by grants from the «Medizinisch-wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien» and «Hochschuljubiläumsstiftung der Stadt Wien», Austria.

Abbreviations: M = monocytes; *MΦ* = macrophages; *CR1, CR2, CR3* = complement receptors; *IL-2R* = Interleukin 2 receptor; *ICAM-1* = intercellular adhesion molecule 1; Bet v I = *Betula verrucosa I; VLA-1* = very late antigen 1; *TcR gamma/delta* = T cell receptor gamma/delta; *LFA-1* = leukocyte function associated antigen 1

radical tumor dissection leads to marked functional disabilities. The revascularized autotransplant of jejunum enables an immediate reconstruction of the defect and is therefore a surgical therapy of choice (1, 2). This kind of treatment is usually supported by local radiotherapy (3). In the past, the results of this technique were evaluated by functional parameters such as swallowing and speaking, the subjective impression of cosmetic corrections (4, 5), and by the frequency of complications (6, 7). Moreover, possible consequences of simultaneous transplantation of gut-associated lymphoid tissue (GALT) should not be ignored. Clinical observation suggests that cells of the GALT in the autotransplant may be involved in defense functions, especially local immunosurveillance against neoplastic cells (K. Ehrenberger, unpublished observations). Since these cells with immunological functions are radiosensitive and may be destroyed by radiotherapy, immigration of new cells («lymphocyte homing») has to be taken into account. Although specialized cells such as macrophages, T cells, B cells and their functions in the GALT are examined in a number of studies (8–10), no information is available at present about changes and characteristics of lymphocytes and mononuclear phagocytes in the autotransplanted and irradiated jejunum.

Considering this lack of data, the surface receptor-equipment and the phenotype of cells with possible defense functions, in particular of the M/MΦ lineage, were investigated.

Patients and Methods

Patients

Group 1 consisted of nine patients (males, aged 38 to 63 years, median 54) with histologically confirmed diagnosis of primary squamous cell carcinoma of the upper aerodigestive tract, one of whom had a relapse (Table 1). All patients were in advanced stages of their disease, graded T4 according to the UICC classification 1987 (11). Six patients revealed evidence of regional lymph node metastases, but none of the patients had distant metastases at the time of surgery. After radical tumor dissection, jejunum was autotransplanted depending on the localization and expansion of the tumor as siphon, patch, tube or as a combination of siphon and patch. Local radiotherapy followed up to 3 months after transplantation (daily dose 1.8 Gray; cumulative dose 50–70 Gray), except in the case of one patient who received chemotherapy (Table 1). Tissue blocks of jejunum were taken at the time of transplantation and biopsies up to 25 months thereafter.

Group 2 consisted of 5 patients (males, aged 52–62, median 55). These patients showed grade T4 of the disease; only one patient was without metastases of the regional lymph nodes (Table 1). Treatment was the same as mentioned in group 1. In contrast to group 1, biopsies of the autotransplanted jejunum could only be taken during the follow up study (up to 50 months after surgery).

Preparation of tissue sections

Biopsies (max. size 5 × 5 mm) and tissue blocks (same size) were snap frozen in liquid nitrogen-cooled methylbutane and stored under liquid nitrogen until use. Serial cryostat sections (6 µm) mounted on poly D-lysine coated glass slides were air dried for two hours and fixed at room temperature for 5 min in acetone, then for 5 min in chloroform and finally for 5 min in acetone. Haematoxilin-eosin stainings were also done routinely for each biopsy.

Table 1. Clinical features of patients

Patient	TU localization	TNM	Histological grading	Irradiation	Mode of transplantation	ΔT[a]
group 1						
1	hypopharynx	T4N2	high	55 Gray	combination	25
2	hypopharynx	T4N2	high	[b]	siphon	24
3	larynx	T4N2	moderate	50 Gray	siphon	17
4	larynx	T4N0	poor-moderate	60 Gray	siphon	12
5	hypopharynx	T4N3	moderate	50 Gray	siphon	11
6	hypopharynx	T4N0	high	55 Gray	siphon	9
7	hypopharynx	T4N2	poor-moderate	60 Gray	siphon	7
8	hypopharynx	T4N0	poor-moderate	60 Gray	siphon	5
9	[c]	rT4N2	high	60 Gray	tube	1
group 2						
1	hypopharynx	T4N3	poor	50 Gray	siphon	50
2	hypopharynx	T4N3	high	55 Gray	combination	22
3	larynx	T4N3	poor-moderate	50 Gray	siphon	19
4	hypopharynx	T4N2	high	50 Gray	siphon	18
5	larynx	T4N0	high	70 Gray	siphon	1

[a] ΔT difference of time in months between transplantation and biopsy
[b] chemotherapy (5 cycles, cis-platin, bleomycin, methotrexat)
[c] status post laryngectomy

Immunocytochemistry

Source and specificity of monoclonal antibodies (mAbs) are listed in Table 2. Appropriate dilutions of mAbs were determined in preliminary experiments. Immunostaining was performed as described earlier either with an alkaline phosphatase/anti-alkaline phosphatase technique (APAAP) (13) or with peroxidase/anti-peroxidase technique (PAP) (14).

To distinguish between $CD4^+$ M/MΦ and other $CD4^+$ cells (mainly T cells), a double immunohistochemical staining was employed. First, mAb Leu4 (CD3) was used to mark all T cells in an indirect peroxidase system (brown reaction product; 15) and staining procedure continued with the respective second mAb Leu3a+b (CD4) using APAAP technique, now staining the helper T cell compartment (mixed brown blue color) and the $CD4^+$ M/MΦ compartment (purely blue color). $CD4^+$ M/MΦ were counted. CD3+ cells were stained in an indirect staining technique (APAAP) and counted. $CD3^+$ cells were also determined by counting $CD3^+$ $CD4^-$ and $CD3^+$ $CD4^+$ cells stained by the above-mentioned double-staining technique. Counts of $CD3^+$ cells did not significantly differ. Controls consisted of an irrelevant mAb of the IgG_1 isotype, BIP-1, directed against Bet v I, the major allergen from birch pollen (16).

Evaluation of positive stained cells and statistical analyses

Cells were counted in five representative areas in a square grid with 100 squares sized 0.04 × 0.04 mm/square; final magnification ×400. Representative areas were selected by examining the whole sections. Consequently, five areas, 2 areas with dense, 2 areas with intermediate and 1 area with weak cellular positivity were chosen for examination. Positive cells were expressed as numbers of immunostained cells per mm^2.

For patients in group 1, the Student's t-test was used to investigate whether changes of numbers of positive cells between the original and the autotransplanted jejunum were significant.

Values of the 14 autotransplanted jejunum were examined for correlation using the Spearman rank correlation.

Table 2. Monoclonal antibodies

Antibody	Subclass	Specificity	Source
AntiLeu4	IgG1	CD3, T cell	BD[a]
Anti Leu3a+b	IgG1	CD4, T subset	BD
Anti Leu2a	IgG1	CD8, T subset	BD
Anti CR1	IgG1	CD35, CR1, gran, MΦ, B	BD
Anti CR2	IgG2a	CD21, CR2, B	BD
Anti HLA-DR	IgG2a	B cells, M/MΦ	BD
Anti HLA-DQ	IgG1	B cells, M/MΦ	BD
Anti HLA-DP	IgG1	B cells, M/MΦ	BD
Anti IL-2 receptor	IgG1	CD25, IL2-R, activated T, B, MΦ	BD
Vim 12	IgG1	CD11b, CR3, NK, gran, MΦ	Knapp et al., 1984
BW 209	IgG1	CD 16, Fc γ R III; NK, gran, MΦ	Behring
3-2	IgG1	CD 64, Fc γ R I, M/MΦ	Medarex
IV.3	IgG2b	CD 32, FC γ R II, M/MΦ, gran, B	Medarex
OKM 5	IgG1	CD 36, M/MΦ	Ortho
27E10	IgG1	Subpopulations of M/MΦ, granulocytes	Zwadlo et al., 1986
RM 3/1	IgG1	MΦ	Zwadlo et al., 1987
25F9	IgG1	mature MΦ	Zwadlo et al., 1985
Identi-T TCR δ1	IgG1	T cell subset (T cell receptor γ/δ+cells)	T Cell Sciences
ACT-T-SET VLA-1	IgG1	CDw49a, α 1 VLA chain, activated T cells, mesangial cells, liver sinusoids, skin fibroblast	T Cell Sciences
Anti ICAM-1	IgG1	CD 54, broad, activat.	Immunotech
Anti IgA1	IgG1	IgA1+cells	BRL[b]
Anti IgA2	IgG2b	IgA2+cells	BD
Anti IgM	IgG1	IgM+cells	BRL
Anti IgG	IgG2b	IgG+cells	BD
Anti IgD	IgG1	IgD+cells	BRL
Anti IgE	IgG1	IgE+cells	Immunotech

[a] Becton Dickinson
[b] Bethesda Research Laboratories

Results

Histology of the autotransplant

The typical structure of the small bowel was still evident, but with extreme shortening and widening of the villi, which were standing close to each other. In the epithelium a reduced number of goblet cells was observed. In the *lamina propria* and in the *submucosa,* a fibrosis and degenerative changes of the vascular system – from teleangiectases up to complete obliterations – was observed, varying between the patients. Nerve cells of the *plexus submucosus* remained unchanged.

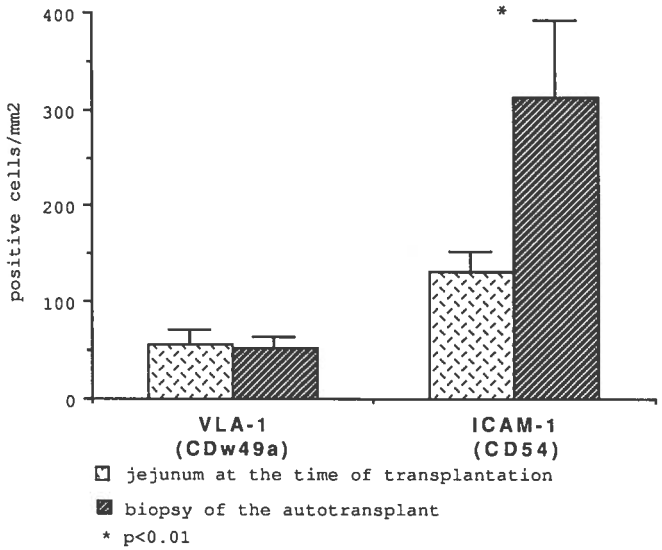


Figure 1. ICAM-1 (CD54) and VLA-1 (CDw49a) antigens expressed on cells in the epithelial layer and the *lamina propria* of the jejunum and the autotransplanted jejunum: Columns show median values ± standard deviations.

T and B cells

After transplantation, B cells as detected by mAb CD21 (CR2; Fig. 5d) increased significantly ($p < 0.01$; Fig. 4) whereas cells bearing other

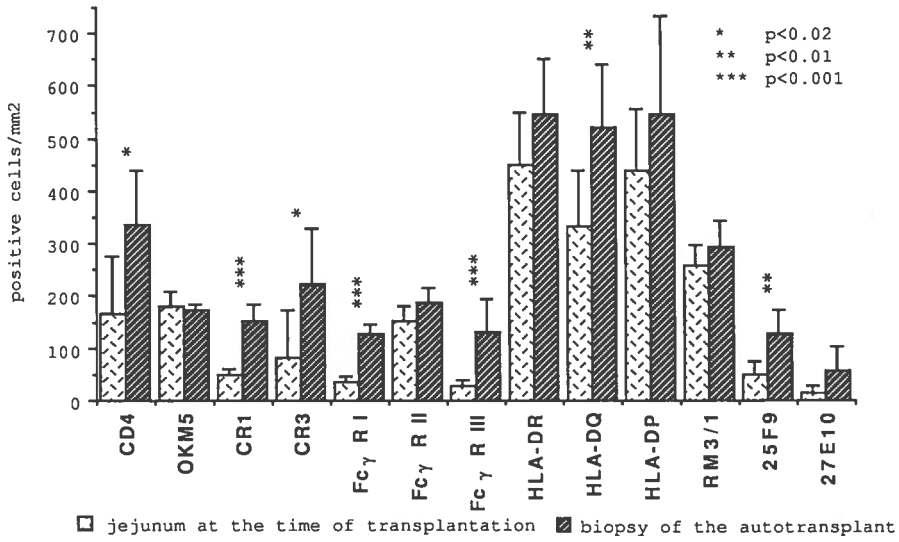


Figure 2. Antigens expressed on cells of the monocyte/macrophage lineage in the epithelial layer and the *lamina propria* of the jejunum and the autotransplanted jejunum: Columns show median values ± standard deviations.

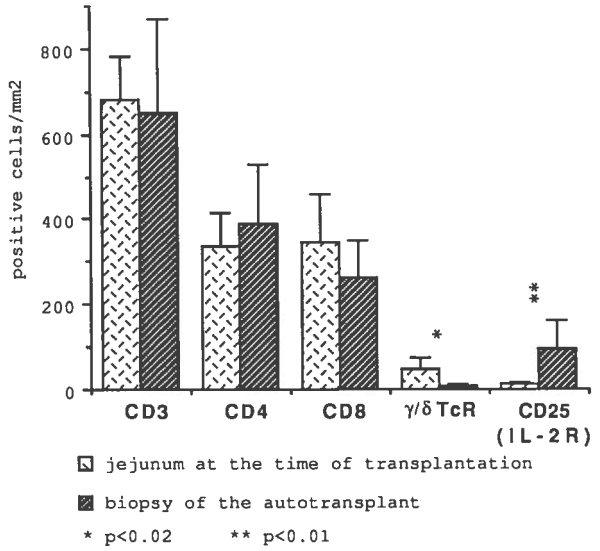


Figure 3. Lymphocyte surface antigens expressed on cells in the epithelial layer and the *lamina propria* of the jejunum and the autotransplanted jejunum: Columns show median values ± standard deviations.

immunoglobulin isotypes decreased. Between the different isotypes, however, subtle differences could be noticed: the number of IgM^+ ($p < 0.01$) and IgA_1^+ ($p < 0.01$) cells decreased significantly. A non-significant tendency for decreased IgA_2^+ and for increased IgD^+ and IgE^+ cells, respec-

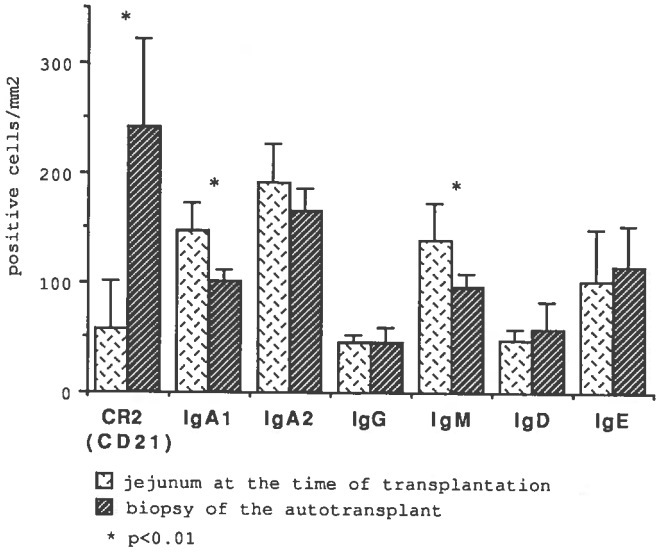


Figure 4. Characterization of B cells in the epithelial layer and the *lamina propria* of the jejunum and the autotransplanted jejunum: Columns show median values ± standard deviations.

tively, was observed. No changes were seen with IgG^+ cells (Fig. 4). Comparing the number of $CD3^+$ cells before and after transplantation (Fig. 5a), T cells showed a tendency to decrease (Fig. 3) and no change in the CD4/CD8 ratio was noticed.

In the autotransplanted jejunum $CD8^+$ cells lost their former position as a layer beneath the basement membrane of the epithelium.

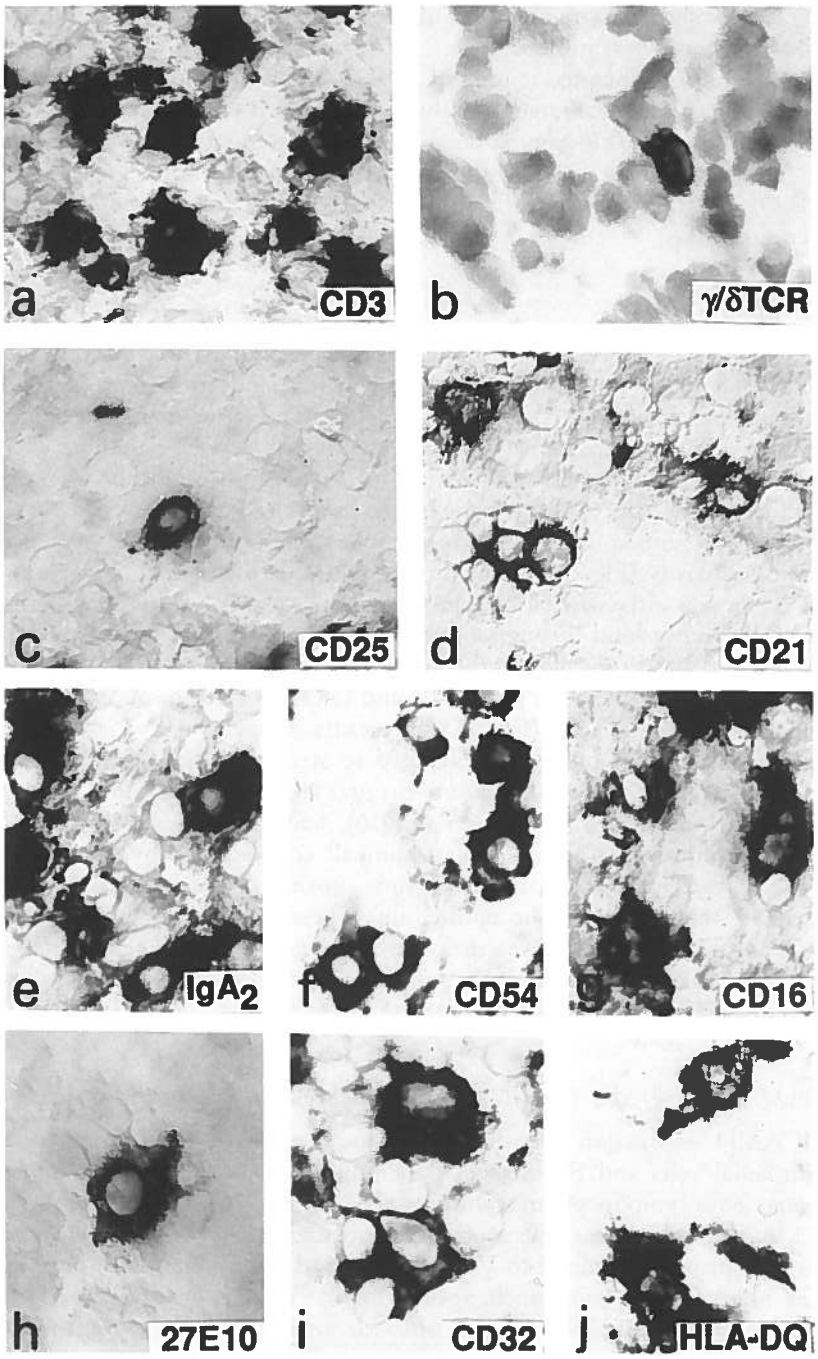
The number of TcR gamma/$delta^+$ cells decreased ($p < 0.01$; Fig. 3; Fig. 5b; Fig. 6a, 6b) in the autotransplant, whereas the number of IL-$2R^+$ (CD25) cell increased significantly ($p < 0.01$; Fig. 3, Fig. 5c) after the operation.

Macrophage markers

Expression of MHC class II molecules on cells with M/MΦ morphology was found not to be altered significantly before and after transplantation with the exception of HLA-DQ expression. The latter increased significantly in the autotransplant ($p < 0.01$; Fig. 2, Fig. 5k). The number of Fc gamma receptor I and III (CD64; CD16; Fig. 5g) positive cells increased significantly in the autotransplanted jejunum (in both cases $p < 0.001$), whereas Fc gamma R II (CD32) positive cells showed only increased staining intensity (Fig. 2, Fig. 5i). In addition, the orientation of Fc gamma R I^+ cells was altered: Before transplantation, weakly positive Fc gamma R I^+ cells were found throughout the *lamina propria* but afterwards these cells were located mainly at the top of the villi. In a similar manner, expression of CR1 (CD35; $p < 0.001$) and CR3 (CD11b; $p < 0.02$; Fig. 6c, 6d) increased significantly (Fig. 2). The picture of few, weakly $CR1^+$ cells – near the *muscularis mucosae* – changed to strongly positive cells, distributed throughout the whole *lamina propria* after transplantation. M/MΦ markers RM3/1 (Fig. 5j), OKM5 (CD36), and 27E10 (Fig. 5h) did not change significantly (Fig. 2). But again, all cells stained by these three markers revealed a loss of their former position as a layer below the basement membrane of the epithelium. These cells were found in the autotransplant dispersed over the whole *lamina propria*. The number of $CD4^+$ and $25F9^+$ cells increased significantly ($p < 0.01$ and $p < 0.02$, respectively; Fig. 2) as compared with the jejunum at the time of transplantation.

ICAM-1 (CD54) and VLA-1 (CDw49a) expression

ICAM-1 expression was found on mononuclear cells, granulocytes, endothelial cells and fibroblasts. Labelling was most prominent on cell clusters with lymphocyte morphology (Fig. 5f, Fig. 6e, 6f). The number of ICAM-1^+ mononuclear cells increased significantly ($p < 0.01$; Fig. 1) in the autotransplant, in contrast to VLA-1^+ cells with lymphocyte morphology, their number remaining unchanged (Fig. 1). VLA-1 positivity was also detected on endothelial cells and smooth muscle cells in the autotransplanted jejunum.


a
CD3
b
γ/δTCR
c
CD25
d
CD21
e
IgA2
f
CD54
g
CD16
h
27E10
i
CD32
j
HLA-DQ

Correlation analyses

Regarding the autotransplanted jejunum, two subpopulations of MΦ with distinct phenotype could be evaluated (Table 3):

1. CR1 (CD35) positivity correlated significantly with the expression of all MHC class II antigens, Fc gamma R II (CD32) and Fc gamma R III (CD16), RM3/1 and with IL-2R (CD25) (Table 3).
2. 25F9 positivity correlated significantly with CD4, HLA-DR, IL-2R (CD25) and with ICAM-1 (CD54) expression.

Discussion

The results of this study suggest that in spite of the partly massive radiotherapy, obviously no significant decrease in the populations of immunocompetent cells took place in the autotransplanted jejunum. When the results were summarized one could even see increased expression of many antigens, which were associated with maturation and/or activation of cells: Other authors showed in other systems that augmented expression of Fc gamma or complement receptors were accompanied by enhanced cellular activity, like increased ADCC (17, 18) or increased cytokine production (19). These changes in the autotransplanted jejunum may be attributed to various factors, such as radiotherapy, the new microenvironment or even the influence of still existing neoplastic cells.

From the literature it is known that resting lymphocytes belong to the most radiosensitive cells (20, 21). Due to these results almost all resting lymphocytes in the autotransplant probably should have been killed by the radiotherapeutic dose of 1.7 Gray per day our patients received. Since lymphocytes have been found in the irradiated autotransplant immediately after the irradiation, one can assume that an instant repopulation and/or local proliferation of surviving resident cells account for the undiminished cell numbers. In contrast to lymphocytes, a higher radioresistance is described for cells of the M/MΦ lineage and for plasma cells (22).

The fact that the number of IL-2R+ (CD25) cells increased (Fig. 3, Fig. 5c) points to an early T cell activation (23), which was obviously not hindered by the preceding irradiation. M/MΦ and cells with dendritic morphology showed a distinct change in as much as certain antigens (Fc

◀ Figure 5. Immunostaining of cryostat sections of autotransplanted jejunum with mAbs directed against leukocyte surface antigens and immunoglobulins: b, h × 640, interference contrast, PAP-technique counterstained with haemalaun; a, c-f, i-j × 640, interference contrast, APAAP-technique counterstained with nuclear fast red. a: section stained with CD3 mAb (T cells); b: gamma/delta$^+$ T cells within the epithelial layer; c: adjacent section to a. stained with mAb against Il-2R (CD25); d: CR2 (CD21$^+$) B cells in the *lamina propria*; e: typically shaped plasma cells positive for IgA2; f: heavily stained clusters of ICAM-1 (CD54$^+$) cells; g-j: cells with typical morphology of macrophages and positive for Fc gamma R III (CD16) (g), 27E10 (h), Fc gamma R II (CD32) (i) and HLA-DQ (j).

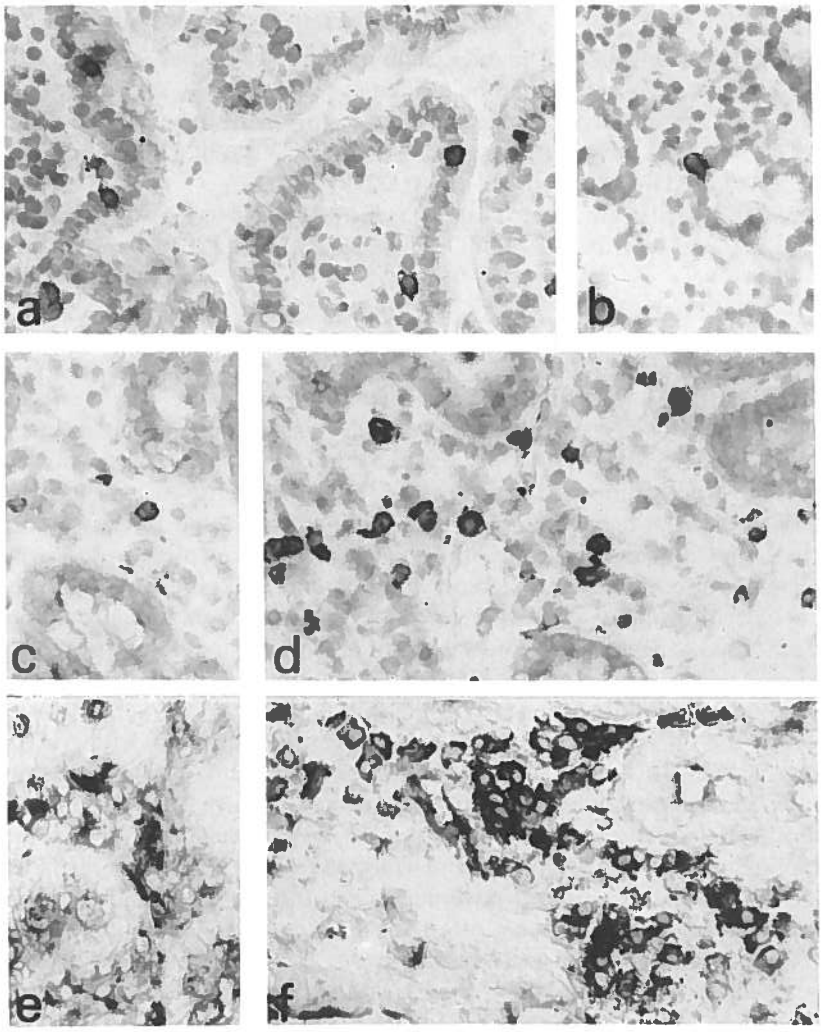


Figure 6. Immunostaining of cryostat sections of jejunum-biopsies a, c, e represent jejunum gained at the time of transplantation; b, d, f biopsies of the autotransplanted jejunum; a-d × 260, interference contrast, PAP-technique counterstained with haemalaun; e, f × 260, interference contrast, APAAP-technique counterstained with nuclear fast red: a, b: gamma/delta$^+$ T cells, in contrast to the jejunum at the time of transplantation (a), where positive cells were also found in the *lamina propria*; note the decrease of positive cells in the autotransplant (b), which are found only scarcely within the epithelial layer; c, d: In the case of CR3 (CD11b$^+$) cells a marked increase could be observed in the autotransplanted jejunum (d) compared to jejunum at the time of transplantation (c). The cells are round with ovular shaped nucleus. e, f: ICAM-1 (CD54$^+$) cells, before transplantation (e) only a few cells expressed this antigen, whereas after transplantation (f) strong positivity was found on a high number of cells, forming mainly clusters.

Table 3. Correlations of antigen expression

mAb	CR1 (CD35)	25F9
CD4+MΦ	0.334^{ns}	0.811^{**}
HLA-DR	0.781^{**}	0.657^{*}
HLA-DQ	0.656^{*}	0.222^{ns}
HLA-DP	0.766^{**}	0.471^{ns}
Fc γ R II (CD32)	0.769^{**}	0.428^{ns}
RM3/1	0.663^{*}	0.301^{ns}
ICAM-1 (CD54)	0.484^{ns}	0.727^{*}
II2-R (CD25)	0.815^{**}	0.7^{*}
25F9	0.502^{ns}	1
27E10	0.334^{ns}	0.659^{*}
Fc γ R III (CD16)	0.638^{*}	0.495^{ns}

ns = not significant
* $p < 0.05$
** $p < 0.01$

gamma RI–III, CR1, CR3, CD4, RM3/1 and 25F9) showed enhanced expression on these cells in the autotransplant (Fig. 2, Fig. 5g, 5i, 5j).

Many studies with M/MΦ demonstrated that continuous culturing of M/MΦ enhanced the expression of Fc gamma receptors (24, 25), CD4 (26), MHC class II antigen (27), complement receptors, 25F9 and RM3/1 antigens. ZWADLO et al. could find that in acute gingival inflammation more $27E10^{+}$ MΦ could be found than $25F9^{+}$ and $RM3/1^{+}$ cells (28). In chronic inflammation, the pattern changed in favor of $25F9^{+}$ and $RM3/1^{+}$ MΦ (29, 30, 31). Thus in the irradiated jejunum we found a pattern similar to chronic inflammation (Fig. 2, Fig. 5h, 5j).

T cells express their T cell receptors as an alpha/beta or as a gamma/delta heterodimer (10). Both subpopulations are present in the gut to a variable degree. We focussed our interest on T cells expressing the gamma/delta chains, because a special role for these cells in mucosal immunity is postulated (32). RAULET (33) could find that these cells do not depend upon MHC restriction in antigen recognition and represent an evolutionary primitive defense mechanism, perhaps similar to the natural killer system in blood. In certain chronic diseases of the bowel (like coeliac disease) (34) the number of gamma/delta T cells increase. In contrast, autotransplanted jejunum showed a significant reduction of these cells (Fig. 3, Fig. 5b, Fig. 6e, 6f). This fact may be due to a different radiosensitivity of the gamma/$delta^{+}$ T cell subpopulation. Also the irradiation and/or the new microenvironment may cause changes of homing receptors, thus leading to the significant decrease.

GALT observed in autotransplanted jejunum showed a higher number of mature B cells (CR2, CD21; Fig. 5d) and also IgD^{+} cells, but a significantly lower number of $IgA1^{+}$ and IgM^{+} cells (Fig. 4). We can only speculate

about reasons for this isotype switch. Cells of the M/MΦ lineage could be influenced by irradiation to produce prostaglandins that may inhibit immunoglobulin production and secretion (35, 36), thus leading to decreased numbers of IgM^+ and IgA^+ cells (37). Furthermore, changes observed in the distribution of $IgA1^+$ and $IgA2^+$ (Fig. 4, Fig. 5e) cells between the jejunum at the time of transplantation and the autotransplant after some months may be due to the different resistance to IgA-specific proteases which are produced by a variety of bacterial species in the new microenvironment (38).

ICAM-1, the ligand of LFA-1, is expressed in variable intensity on a wide spectrum of cells (39, 40). This molecule belongs to the family of adhesion molecules which regulate leukocyte adhesion and migration to areas of inflammation and/or reparation (41). ICAM-1, the representative of the adhesion molecule family in this study, showed augmented expression on mononuclear cells as well as on endothelial cells after transplantation and irradiation (Fig. 1, Fig. 5f, Fig. 6e, 6f), a fact, which would help immunocompetent cells to immigrate and further facilitate cellular interactions (42). In contrast, the numbers of VLA-1+ cells (VLA – a member of the integrin family) remained unaltered (Fig. 1) and thus suggests a different regulation mechanism for these antigens. VLA-1 positivity of T cells is found in late stages of T cell activation and is involved in the interaction of T cells and tissue matrix (collagen Type IV) (43, 44).

Finally, a possible systemic influence of local radiotherapy has to be considered. The controversy in literature concerning the distribution of lymphocyte subsets in the peripheral blood after local radiotherapy may be due to different body regions, causing different changes of distribution of blood lymphocyte subsets (45). Following local irradiation, peripheral blood lymphocytes display altered functional states to various agents. These functional impairments lasted for years and were also detected in patients suffering from head and neck cancer and receiving local radiotherapy (46). The observed immunohistochemical staining patterns have to be considered with regard to altered functional capacities.

In one case (pat. 8; data not shown), we were able to obtain three biopsies in continuous intervals over half a year. These results suggest a time dependency of changes.

In the same way, the chemotherapy of patient 2 (see Table 1) may exert an influence, but due to the lack of data it is impossible to ascertain changes different to those observed in the irradiated autotransplanted jejunum.

The main purpose of irradiation, namely the elimination of putative remaining neoplastic cells, seems to be often not relevant. Additionally, there are indications for suppressive influence of irradiation, raising the question of the benefit of radiotherapy for the patient. Transplanted GALT, although irradiated, still has immunological capacities as detected by an increase of cells expressing activation and/or maturity associated antigens. It would be certainly of great interest to differentiate and analyze the different influences, which are probably exerted by irradiation, altered

microenvironment and possibly remaining neoplastic cells and may influence defense mechanisms in the autotransplanted jejunum.

Acknowledgments

We thank Dr. W. Knapp (Vienna; Austria) and Behring Austria for providing the antibodies VIM 12 and BW209, respectively.

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