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The Effect of Antigen Stimulation on Splenic Transplants

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Summary

The present paper deals with the regeneration of splenic tissue after autologous transplantation. Control and transplanted rats (60 days after operation (10^6 cells per injection). The effect of a primary response was studied by a single injection, long-lasting bacteraemia was imitated by 5 injections in weekly intervals. Spleens and transplants were investigated by flow-cytometry and immunohistochemistry. Additionally, the proliferation activity and the specific antibody production against *Escherichia coli* proteins were tested. Flow-cytometric analysis showed altered behaviour of T-helper cells and B-cells in transplants following a primary response, whereas in the multiple injection group a difference between the splenic and transplant response was restricted to macrophages and MHC II^+ cells. The results of the morphometric analysis revealed that the cellular composition of unstimulated transplants was very similar to that of the spleen with some subtle alterations. Only the marginal zone showed more striking differences concerning the homing of several cell classes. Under stimulatory conditions, these subtle alterations became more drastic so that CD5+ cells, B-cells and macrophages responded in an abnormal manner in both groups. The analysis of thymidine kinase disclosed decreased activity in the spleen after weekly antigen stimulation. The stimulation index of all transplant groups was significantly lower than that of the spleen. The specific antibody (IgG) production after a single immunization was highest in the transplant group. All groups responded after the multiple challenge. In conclusion, the results demonstrate that splenic transplants differs in several, but subtle aspects from normal splenic tissue. The main reason for most of these alterations may be a slightly misguided recirculation and/or homing of cells.

Key words
Splenic autotransplantation – Immunohistology – Flow-cytometry – Antigen stimulation – Proliferation

Introduction

Regeneration of immunocompetent organs such as the spleen (or lymph nodes) represents a phenomenon with interesting aspects concerning both clinical and basic immunological research. Although used in more than 400 cases, splenic autotransplantation in man (Buntain and Gould 1985, Pisters and Pachter 1994), as an alternative to splenectomy after massive splenic rupture, is still controversial. One reason for this is that most of the published data dealt with blood flow, clearance and protection. Furthermore, studies concerning the protective effect of regenerated splenic tissue were performed using different species of various ages, providing different doses and application routes (for review see Pabst *et al.* 1991).

In recent papers describing the effect of the target tissue on the immunoarchitecture, cellular functions and expression of cytokine genes, we found that despite the reorganization of splenic compartments subtle morphological and functional alterations had occurred in the transplants (Liaunigg *et al.* 1992, Thalhamer *et al.* 1989). In addition,

differences between replants at different implantation sites were detected. These results indicated changes of the functional homing and/or recirculation of cells in transplants and an additional dependence on the microenvironment of the implantation site.

In the present paper, we investigated possible changes of the homing within compartments of the spleen and transplants after intravenous stimulation with *Escherichia coli*. This was done by measuring the cell density of lymphocyte and macrophage subsets within the red pulp, marginal zone, periarteriolar lymphoid sheath and follicles with and without antigen stimulation. Comparing the splenic and transplant reactions should enable us to find alterations in the functional homing of splenic transplants compared to the spleen.

Materials and Methods

Animals

Young Lewis rats (4–6 weeks) of either sex kept under conventional conditions were used. The animals were grouped in the following way:

Control animals: non-stimulated, non-operated (n=8); non-stimulated, splenectomized (n=8), non-stimulated, autotransplanted (n=8).

Once immunized animals: non-operated (n=6); splenectomized (n=5); autotransplanted (n=5).

Weekly immunized animals: non-operated (n=4); splenectomized (n=5); autotransplanted (n=6).

Sixty days after transplantation the regenerated splenic nodules were removed and from each transplant one part was immediately prepared for the flow-cytometric analysis and a second part was frozen and stored in liquid nitrogen for the histological and proliferation analysis. Because three splenic slices were implanted into each animal of each group, the total number of individually investigated transplants was 57.

Surgery

After anaesthesia with Ketavet (Parke-Davis, Berlin, FRG) and Rompun (Bayer, Leverkusen, FRG) in Ringer lactate solution (4:1:5, 2 μl per gram body weight, injected subcutaneously), splenectomy and autotransplantation were performed as previously described (Thalhamer *et al.* 1992)

Immunization and preparation of serum

After light sedation of the rats with diethyl ether, 10^6 living *E. coli* cells were injected into the tail vein (this dose is neither pathogenic nor induces anaphylactic reactions in the animals). Three days after the primary immunization (or after the last injection), the animals were sacrificed, and the serum was produced as described by Hammerl *et al.* (1988)

Antibodies

The following antibodies (mouse anti-rat, Serotec, Oxford, UK) were used: W3/13 (thymocytes, T-cells); W3/25 (T_{helper}-cells, CD4), OX-8 ($T_{supp/cyt}$-cells, CD8), OX-12 (kappa/light chain), OX-6 (Ia-antigen, dendritic cells), ED-1 (macrophages, monocytes and dendritic cells) as described by Barclay (1981). The second antibody (polyclonal goat anti-mouse Ig peroxidase antibody) was obtained from Biorad (Richmond, USA).

Immunohistology

Blocks of fresh splenic tissue and whole splenic transplants were frozen and stored in liquid nitrogen. Cryostat sections and staining was performed as previously described (Liaunigg *et al.* 1992).

Preparation of cell suspensions

Spleen and transplants were minced in phosphate buffered saline, pH 7.2 (PBS) producing a splenic pulp. In order to sediment aggregated cells, the suspension was transferred into a test tube for 10 min, and the supernatant (monodisperse cell suspension) was washed 3 times in 10 ml PBS. The viability of the cells was determined by trypan blue exclusion.

Flow-cytometry

The fluorescence background was determined with a control sample that was treated with the second antibody alone [fluorescein isothiocyanate(FITC)-conjugated goat-anti-mouse-antibody]. The data of 5000 cells from each sample were stored and analysed, and the percentage of positive cells was calculated using windows-setting as described (Thalhamer *et al.* 1992).

ELISA

The specific antibody response was measured by ELISA using a commercial ABTS Peroxidase EIA Substrate Kit (Biorad, Richmond, USA). The sera of the animals of each experimental group were pooled and added (1:2500 dilution) to microtiter wells (Nunc, Denmark) coated with 20 μg *E. coli* crude extract. Blocking and washing were performed as described by Osawa *et al.* (1986). As second antibody sheep anti-rat IgG coupled with horseradish peroxidase (1:1000 dilution) was used and stained for peroxidase activity with 2,2'-azino-di(3-ethylbenzithiazo-6-sulfonic acid) and hydrogen peroxide. The resulting dye was measured on an EIA-reader (Titertek Multiscan, Flow Laboratories, USA) at 415 nm.

Thymidine kinase assay

A 10 % homogenate of splenic and autotransplant tissue was prepared in 100 mmol Tris-HCl buffer, pH 8.0, containing 1 mmol $MgCl_2$ and 0.1 mmol PMSF. Subsequently, the homogenate was cleared by centrifugation at 1000 x *g* and the obtained

supernatant spun down at 104 000 x *g*. This second supernatant was carefully aspirated avoiding the top layer containing fat, aliquots of this sample were added to the incubation buffer (containing ATP, $MgCl_2$ and ^{3}H-thymidine) and thymidine kinase activity was measured as described by Zieve *et al.* (1985). Counting was done in a liquid scintillation counter (LKB 1217 Rackbeta, LKB, Sweden) and the results were expressed as disintegrations per minute per milligram of protein.

Analysis of data

Quantitative analysis of the immunohistological sections was performed by the point-counting method (Weibel 1979). An ocular square grid (the grid represented 100 μm^2 at 1000x magnification) was used for counting the antibody-positive cells in each compartment. The results were expressed as the mean ± standard deviation, unless stated otherwise. Significant differences between groups were determined by Mann-Whitney-two-sample t-test (software NCSS 5.1, Unisoft, Augsburg, FRG).

Results

Morphology

In general, splenic regeneration in the rat is completed within 40-50 days after transplantation. Therefore, all transplants (60 days old) showed the typical nodule form and dark red colour of splenic tissue including little white spots indicating areas of red and white pulp. Histology and immunohistology revealed the existence of splenic compartments such as red pulp, follicles, periarteriolar lymphoid sheaths (PALS), marginal zone and a spleen-like distribution of different cell classes and subpopulations (data not shown – see Liaunigg *et al.* 1992, Thalhamer *et al.* 1992).

Cell composition of spleens and transplants

Figure 1 represents a flow-cytometric comparison of spleen and transplant cells of control, once immunized and weekly immunized animals. The following two aspects of these experiments should be considered:

a) The control groups permit a comparison of the cellular patterns of spleen and splenic transplants, thus giving general information about transplant development with respect to the cellular composition.
b) The stimulated groups of spleen and transplants provide the opportunity of analyzing the functional behaviour of transplants under stimulatory conditions compared to the normal splenic reaction.

The most striking differences between the cell composition of spleens and 60-day-old splenic transplants are restricted to T- and B-cells. In contrast to the spleen, splenic transplants possess more B- and less T-cells. Macrophages are slightly reduced, but MHC-II$^+$ cells are significantly raised in transplants. This indicates that the MHC-II$^+$ (Ia) molecules of B-cells are responsible for the detected increase.

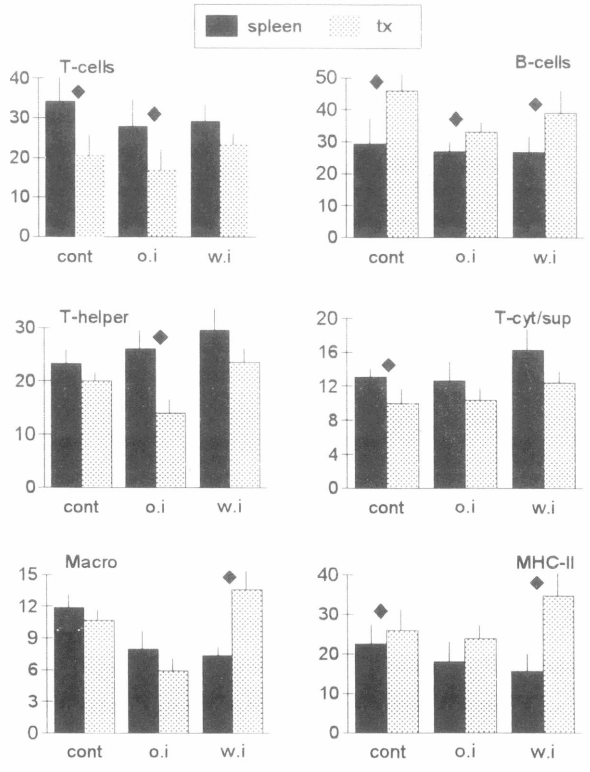

Fig. 1. *Flow-cytometric analysis of lymphocytes and macrophages. The abscissa represents the control values (cont) of spleens (spleen) and transplants (tx) as well as values of once immunized (o.i.) and weekly immunized (w.i.) groups. The ordinate shows the percentage of labelled cells within the leucocyte window. T-cells = W3/13$^+$ cells (total T-cells), B-cells = kappa/light chain$^+$ cells, T-helper = CD4$^+$ T-cells, T-cyt/sup = CD8$^+$ T-cells, Macro = macrophages, MHC-II = Ia$^+$ cells. Rhombi indicate significant differences between spleen and transplants ($p<0.05$).*

Beyond these cellular differences, several alterations could be measured in transplants under "working" conditions. B-, T-helper cells and macrophages seem to be mainly affected by antigen stimulation. Upon immunization (both single and multiple injections), the spleen reacts with no significant changes of the B-cell number, whereas in transplants the number of B-cells is reduced (more strikingly in the group with a single injection of antigen, $p<0.05$). The reaction of total T-cells in transplants compared to the spleen was very similar for both immunization groups. However, T-helper cells of transplants showed a drastic decrease after a single injection. In contrast to this, macrophages and MHC-

II$^+$ cells decreased significantly after multiple injection in the spleen but doubled in the transplants, the reaction to a single injection being comparable to the spleen. In this case, the MHC-II$^+$ cells reflect the behaviour of the macrophages and not the B-cells.

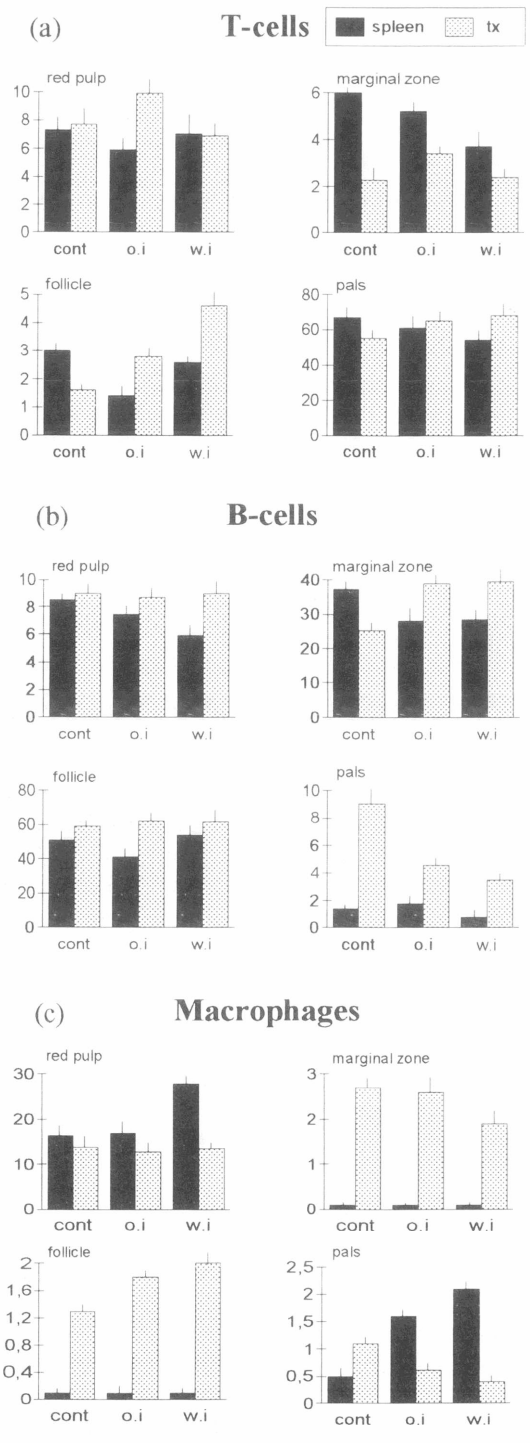

Fig. 2. *Morphometric analysis of T-cells (Fig. 2a), B-cells (Fig. 2b) and macrophages (Fig. 2c) in splenic and transplant compartments. The abscissa is defined as in Figure 1, the ordinate shows the percentage of labelled cells/100 μm^2. Significant differences are mentioned in the text.*

Morphometric analysis of splenic and transplant compartments

Figure 2a shows the density (percent of positive cells/100 mm^2) of total T-cells under the different experimental conditions in all four splenic compartments. As in Figure 1, again the two aspects (general similarity or difference between spleen and transplants and spleen-specific or transplant-specific functional alterations after stimulation) can be distinguished.

Significant differences between spleen and transplants concerning the T-cells are restricted to the marginal zone and follicles ($p<0.05$). Both compartments show a strikingly decreased number of this cell class in the transplants. As a consequence of the single immunization, control spleens react with a significant decrease of the cell density in the red pulp and follicles ($p<0.01$). In contrast, these compartments revealed an opposite behaviour in transplants after a single injection ($p<0.01$). The drop of T-cells in the marginal zone and PALS proved not to be significant, however, the cellular increase in transplants was ($p<0.01$). Multiple injections of antigen elicited significant functional differences only in the marginal zone, the cell concentrations of which was lowered in the spleen but not the transplants and follicles, which showed a drastic increase of T-cells restricted to the transplant group.

Figure 2b represents the density of B-cells. There was more than a fivefold increase in the number of B-cells in the transplant PALS as compared with control spleens ($p<0.001$), whereas the number of these cells was reduced in the marginal zone. After a single injection of the antigen, the marginal zone and PALS again reacted in an abnormal way. The cell density of the splenic marginal zone decreased, whereas it increased in the transplants and the opposite reaction occurred with respect to the splenic and transplant PALS. A similar defect of the marginal zone and PALS was also detected in the groups with multiple injections.

Drastic differences between control spleens and the transplants could be demonstrated by the quantification of ED-1$^+$ macrophages (Fig. 2c). In contrast to the splenic marginal zone, follicle and PALS, which include only very few macrophages of this type (most of the marginal zone macrophages are ED-3$^+$, whereas follicles contain ED-1$^-$ follicular dendritic cells), the transplant compartments showed a strikingly higher density of these cells ($p<0.01$). The spleen-specific reactivity of the compartments was altered only in the multiple immunization group in the red pulp and concerned both groups in the PALS. Splenic reaction induced the increase of macrophages, whereas transplants showed no reaction or a decrease, ($p<0.01$) for all the differences.

Cell proliferation

Cell differentiation, clonal expansion and permanent cell-to-cell interactions not only occur during immune responses but they are also important prerequisites for maintaining a functional microenvironment of complex immunocompetent organs such as the spleen. Thymidine kinase (TK), a key enzyme of the "salvage" pathway producing the essential DNA precursor TTP, was used to measure the rate of cellular activity of splenic and transplant tissue. This was possible because a direct relationship between the rate of cell division and the activity of thymidine kinase is assumed. Figure 3 shows a diagram of the TK-activity (expressed in units/mg protein) of spleens and transplants. The control (untreated) spleens revealed the highest values. Single immunization slightly decreased the enzyme activity (statistically not significant), but multiple injections led to a striking alteration of the proliferative activity. Significant differences were detected between the unstimulated splenic and transplant tissue indicating alterations of the transplant microenvironment. Neither single nor multiple immunization had a significant effect on TK activity in the transplants.

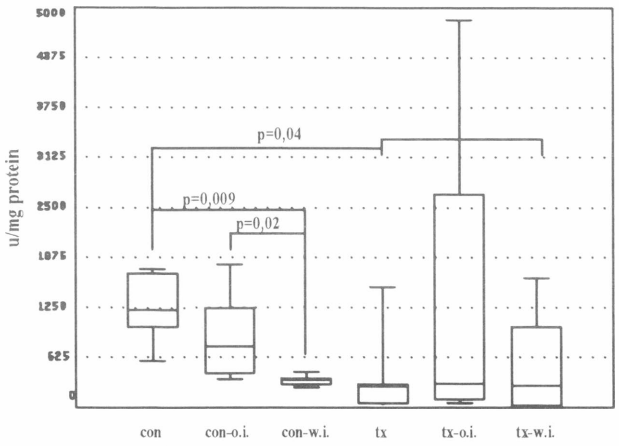

Fig. 3. *Box-blot analysis of the thymidine kinase activity in spleens and transplants. The horizontal line inside the boxes denotes the 50 % fractile value (median), borders of the boxes are at 25 and 75 % fractile values and the crossbar shows the 10 or 90 % fractile values. Con = spleen, tx = transplant, o.i. = once immunized, w.i. = weekly immunized. Significant differences are indicated by connecting lines between several groups.*

Specific antibody production

Serum antibodies directed against an *E. coli* protein extract were detected by ELISA and both immunized groups were tested. Additionally, groups of splenectomized animals (control and immunized) were investigated (Fig. 4). The results are expressed as experimental values minus background values of unstimulated animals.

The antibody titre of control animals was increased significantly only after multiple injections of antigen, whereas transplanted animals reacted with a striking increase even after the first dose of antigen. Splenectomized animals showed no reaction after a single immunization, however, showed the highest values of all groups after multiple injections.

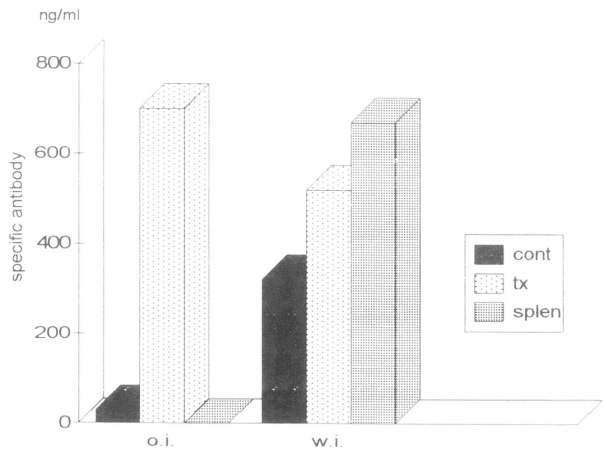

Fig. 4. *Comparison of the specific antibody production under different stimulation conditions (once immunized [o.i.] and weekly immunized [w.i.]) in control (cont), transplanted (tx) and splenectomized (spleen) animals. The concentration of the antibody is given in ng/ml serum.*

Discussion

As has been reported in several papers in the past decade, autotransplanted splenic tissue regenerates well in rodents (for review see Pabst *et al.* 1991). We also found correct reorganization of all splenic compartments 60 days after implantation. The major objective of the present study was to characterize some functional cellular processes directly in transplants and to compare them to the reaction of normal splenic tissue.

Whole *E. coli* cells were chosen as antigen because they are known to be strong immunogens for mammals but are only weakly pathogenic with the doses used. In addition, these bacteria induce a broad spectrum of different immune responses including activation by lipopolysaccharide, a component of the bacterial wall, which mimics a typical complex immune reaction *in vivo* involving the whole organism. A

primary response and long-lasting bacteraemia were imitated by the two immunization schedules.

A detailed picture emerged from the flow-cytometric and morphometric analysis of spleens and transplants. One of the principal differences between normal splenic and regenerated splenic tissue is the lower amount of nucleated cells in the transplants. Even a light-microscopical investigation reveals a dominating red pulp and a decreased size of the white pulp. Especially the number and size of PALS are reduced. This can be seen also upon comparing the flow-cytometric data of control spleens and transplants which demonstrate that T-cells and their subsets are reduced in the transplants. In contrast, the proportion of B-cells (and MHC-II$^+$ cells which also include the B-cells) is raised in the transplants. These results point to a subtle alteration of the lymphocyte homing and/or recirculation processes in splenic transplants. Under stimulatory conditions, these subtle defects of the microenvironment seem to become more obvious because, at least in the primary response, several cell classes exhibit abnormal behaviour in the transplants. However, in long-lasting bacteraemia, differences between spleen and transplants are restricted to the reaction of macrophages and MHC-II$^+$ cells. This indicates the minor involvement of splenic and transplant tissue in secondary responses against bacterial cells.

Because flow-cytometric data can only describe events without considering the complex three-dimensional immunoarchitecture and compartments which are a prerequisite for immunological functions, morphometric analysis was necessary to provide more information about the cellular composition and behaviour. The results of these experiments confirmed that splenic transplants are altered in many ways. However, before discussing the details, the subtle nature of these differences must be emphasized. In general, the cellular composition of the different investigated cell classes within their main compartments is very similar between spleen and splenic transplants (e.g. T-cells and subsets in PALS, B-cells in follicles and macrophages in the red pulp). From this it can be concluded that the regenerated microenvironment works correctly for the major pathways of cell homing and recirculation. Recent studies support this assumption by demonstrating several spleen-specific functions in transplants (for review see Pabst *et al.* 1991). The only compartment which seems to be altered with respect to the homing of several cell classes is the marginal zone. Because this compartment represents the main entrance and distribution route for recirculation and homing of cells, some of the detected disproportions may be the result of a subtle misguiding of certain cells into the wrong compartments. However, even a reduced number (50 %) of T-$_{supp/cyt}$ cells in follicles (data not shown) may have only marginal effects on transplant functions because the normal amount of these cells in a follicle is 3 %. On the other hand, functional aberrations cannot be excluded because of the drastic disproportions (between splenic and transplant compartments) of B-cells in PALS and macrophages in the marginal zone and follicles. The high number of macrophages in these transplant compartments may be leftovers of the intensive phagocytosis of necrotic cells in the first days of regeneration. Concerning the hypothesis that macrophages partly serve as guides for recirculating cells in the splenic tissue, the transplant-specific alterations of this cell class may be responsible for the various subtle disproportions in transplant compartments.

Therefore, the question arose if these subtle changes in transplants result in functional alterations during the course of antigen stimulation. The approach to this problem was to measure the specific changes of cellular composition within the compartments of the spleen and to compare these functional values with the transplants. A typical primary splenic response against whole *E. coli* cells three days after injection can be characterized by a complex combined influx and efflux of cells within the compartments. However, it must be emphasized that an increased number of cells within one compartment does not necessarily mean immigration, but can also result from cell proliferation. On the other hand, a decreased number of cells can be interpreted as emigration of cells or loss of specific markers during differentiation (the latter can be excluded for the investigated markers like CD5, CD4, CD8, kappa/light-chain and ED-1). Nevertheless, any change of cell density within a certain compartment following immunization reflects spleen-specific reactions which may be essential in the course of an immune response.

At the peak of a primary immune response (which is about 3 days after injection), the most prominent cell movements are a decrease of B-cells in the marginal zone, an increase of T-$_{helper}$-cells (data not shown) in most compartments and an increase of macrophages in the PALS. This reflects the known activities of immunocompetent cells during a primary response including the raised antigen presentation and induction of T-cell help which seems to involve all splenic compartments. Follicular B-cells play only a minor role in the primary response, however, mature B-cells and plasma cells move to the outer PALS, which is indicated by the increase of kappa/light chain$^+$ cells in this compartment.

Transplants during the primary response show some changes of these kinetics. For example, T-cells exhibited contrary behaviour in nearly all compartments. The distribution of B-cells and macrophages was also altered in some compartments. These results clearly point to the fact that the subtle immunoarchitectural alterations in transplants have more striking consequences under stimulatory

conditions. Furthermore, similar effects could be detected in the multiple injection group. The B-cell distribution in the marginal zone, T-cells in follicles and especially macrophages in the red pulp and PALS of transplants reacted in an abnormal way after stimulation. However, calculation and comparison of the misguided cells in transplants reveals that the described alterations refer to only a small percentage of the total cell number. This can be demonstrated by the alterations in the number of T-cells in the follicles, which is decreased from 3 to 1.7 % in the spleen and increased from 2.8 to 4.3 % in the transplants. Compared to the total number of cells involved in splenic reactions, this minimal change may be within the tolerated range and be without any functional consequences. In contrast, the majority of cells from the transplants showed a correct homing-behaviour (e.g. for B-cells in the follicles, which contain about 60 % of the total B-cells and T_{helper} cells in the PALS and marginal zone, which contain 60 % of the total T_{helper} cells).

The measurement of thymidine kinase activity revealed interesting results. The values of untreated splenic tissue were the highest of all groups and stimulation decreased the enzyme activity, especially in the weekly immunized group. A possible explanation for these unexpected reactions may be the emigration of activated cells into the recirculating pool of cells and into other immunocompetent organs of the organism. This would be in agreement with the conventional picture of cellular responses in immunological organs. Activation and cell differentiation takes place in the microenvironments of the specific tissues (e.g. the spleen and lymph nodes) and thereafter these cells (carrying the potency to react against foreign antigen) are spread over the whole organism. In this context the striking reduction of the enzyme activity of weekly stimulated splenic tissue can also be interpreted. Secondary and booster reactions against bacterial cells involve primarily different RES-systems and the spleen plays only a subordinate role here. No activation processes occur in the splenic tissue and, moreover, recirculation of activated cells may be guided preferentially to the RES-systems which are involved in the removal of bacteria.

On the other hand, the results also document that the spleen needs to proliferate constitutively, even under non-stimulatory conditions, to maintain its functions (and probably its complex three-dimensional architecture). The upkeep of a complex and dynamic structure including regulated homing and recirculation needs intensive cell-to-cell interactions, production of regulatory molecules and activation processes. This point of view is also supported by data on the expression of cytokines in unstimulated splenic tissue, which also constitutively express several cytokine mRNAs (Tovey *et al.* 1988).

Unstimulated transplant tissue shows a significantly decreased activity of thymidine kinase indicating a more "passive" microenvironment. During a primary response the proliferation is not significantly changed. However, the values are scattered over a wide range of reactivity. This fact indicates that splenic autotransplants of a certain age are rather heterogeneous concerning their functional state and thus react with different kinetics. Weekly administration of cells also induced no significant difference and the reaction was more homogeneous than in the primary response. However, compared to the spleen group, which was extremely homogeneous, the transplants elicited a more scattered response indicating that some transplants also proliferate in secondary and booster reactions.

The evaluation of the specific IgG antibody titre against *E. coli* antigens was additionally done in splenectomized animals in order to characterize a possible effect of splenic transplants. In the control group, a primary response elicited no significant increase of the titre because this type of response primarily involves antibodies of the IgM class. A prolonged multiple injection of *E. coli* cells led to a significant elevation of specific antibodies. Following primary stimulation, the specific antibody titre of transplanted animals was strikingly increased, whereas the group with multiple injections was comparable to the spleen. At present, this high titre of specific IgG antibodies seen three days after injection of the bacterial cells cannot be satisfactorily explained. Therefore, we can only speculate that the microenvironment of the regenerated splenic tissue may provide some unknown stimuli for an early class switch. In contrast to the transplanted group, splenectomized animals showed no reaction after a primary stimulus but responded with a drastic increase of specific antibodies after multiple injections. Because the spleen contains a large number of $T_{supp/cyt}$ cells, which play an important role in restricting an immune response, we assume that the high production rate of antibodies may be the consequence of the missing regulation of splenic tissue. The data demonstrate that a small amount of transplanted tissue affects even very complex *in vivo* responses of the whole system. However, at present we cannot discern if the detected effects have positive or negative consequences for the organism.

As conclusion of the present results it must be stated that despite several, but in principle subtle alterations, transplanted splenic tissue in the greater omentum resembles normal splenic tissue, especially concerning the structure and distribution of different cell classes. A work load elicited some functional alterations of transplants with still unknown consequences for the whole organism. As known from previous studies, transplants are able to fulfill a number of splenic functions in a correct manner,

therefore it can be assumed that the detected effects (or defects) may only have marginal influences.

Beyond these aspects the splenic autotransplantation offers an excellent model system to investigate questions concerning the development and function of complex immunocompetent organs and may be a model for the transplantation of other immunologically active tissues.

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Reprint requests

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