The Interaction Mode of Premalignant Schwann and Immune Effector Cells during Chemically Induced Carcinogenesis in the Rat Peripheral Nervous System Is Strongly Influenced by Genetic Background

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Abstract

Contrary to rats of the highly sensitive inbred strain BDIX, BDIV rats are resistant to the induction of malignant schwannomas by N-ethyl-N-nitrosourea, arising predominantly in the trigeminal nerves. A point mutation of the neu/erbB-2 gene diagnostic of N-ethyl-N-nitrosourea–induced rat schwannomas is an early marker of Schwann precursor cells at high risk of subsequent malignant transformation. Neu-mutant cells initially arise at a similar frequency in sensitive and resistant animals. However, these cells disappear from the trigeminal nerves of resistant rats while giving rise to highly malignant schwannomas in susceptible animals. The resistance of BDIV rats obviously includes mechanisms to recognize and eliminate premalignant cells. The involvement of a cellular immune response was investigated in trigeminal nerves of both strains at different times after neonatal carcinogen exposure. An inflammatory reaction involving sequentially CD4+ macrophages and T helper cells, CD8+ cytotoxic T cells, and ED1+ and ED2+ macrophages was detected as a consequence of N-ethyl-N-nitrosourea treatment as early as postnatal day 40, briefly after the emergence of premalignant neu-mutant Schwann cells. It persisted throughout the observation period (40-250 days). However, there were no gross differences in immune cell counts between tumor-susceptible and tumor-resistant rats, except for a moderate increase of ED2+ macrophages in N-ethyl-N-nitrosourea–treated BDIX rats only. Differential interactions of immune effector cells with premalignant Schwann cells may thus be involved in genetically determined tumor susceptibility or resistance, which could include functional differences of immune effector cells and/or a differential capability of premalignant Schwann cells to escape or counteract the cellular immune response. (Cancer Res 2006; 66(9): 4708-14)

Introduction

Individual cancer risk in humans is determined by complex interactions between germ line genetic variance and levels of exposure to environmental carcinogens or tumor promoters. It has long been known that individuals exist who do not develop cancer in spite of a long history of mutagen exposure, such as heavy smoking. This intrinsic resistance to the effects of carcinogens is likely to depend on the genetic background. Up to now, little is known about gene variants predisposing to cancer or mediating cancer resistance as well as about effector mechanisms of susceptibility and resistance. Investigations aiming at unraveling biological mechanisms of human cancer resistance are confined to animal models because individual exposure to carcinogens is usually unknown in humans, and early premalignant lesions likely to progress to full blown malignant phenotypes are not detectable due to their small cell number and unpredictable location and/or the lack of criteria for their unequivocal identification.

Depending on their genetic background, inbred rodent strains exhibit a broad spectrum of sensitivity towards the induction of malignant tumors by specific carcinogens, ranging from high susceptibility to almost entire resistance. Rats of the inbred BD strains provide a suitable model for the study of resistance to chemically induced carcinogenesis in the peripheral nervous system (PNS) and central nervous system. These strains exhibit differential sensitivity towards the induction of neural tumors by prenatal or perinatal pulse exposure to N-ethyl-N-nitrosourea. Thus, BDIX rats develop malignant schwannomas, predominantly of the trigeminal nerves, with an incidence >85%, whereas BDIV rats are entirely resistant. A transversion mutation at nucleotide 2012 in the transmembrane region of the neu/erbB-2 gene is likely to be the initial event in N-ethyl-N-nitrosourea–induced schwannoma development in sensitive strains of rats. This mutation is diagnostic of N-ethyl-N-nitrosourea–induced rat schwannomas and in the process of neuro-oncogenesis, it characterizes a subset of immature Schwann cells that are mainly located near the brain-nerve junction and exhibit unrestrained proliferative activity in contrast to their differentiating wild-type counterpart cells. neumutant Schwann precursor cells are, therefore, at high risk of progressing towards the expression of fully malignant phenotypes. Knowing precisely the prospective location of the future tumor and being able to recognize and pursue premalignant cells (based on the diagnostic mutation) provides us with the possibility of analyzing early and intermediate stages of carcinogenesis beginning at day 30 after N-ethyl-N-nitrosourea exposure or even earlier, depending on the detection method.

In an attempt to uncover the biological mechanisms leading to tumor resistance in BDIX rats, we have previously quantified neu-mutant Schwann cells in BDIX trigeminal nerves and in trigeminal nerves of both strains at different times after neonatal carcinogen exposure. An inflammatory reaction involving sequentially CD4+ macrophages and T helper cells, CD8+ cytotoxic T cells, and ED1+ and ED2+ macrophages was detected as a consequence of N-ethyl-N-nitrosourea treatment as early as postnatal day 40, briefly after the emergence of premalignant neu-mutant Schwann cells. It persisted throughout the observation period (40-250 days). However, there were no gross differences in immune cell counts between tumor-susceptible and tumor-resistant rats, except for a moderate increase of ED2+ macrophages in N-ethyl-N-nitrosourea–treated BDIX rats only. Differential interactions of immune effector cells with premalignant Schwann cells may thus be involved in genetically determined tumor susceptibility or resistance, which could include functional differences of immune effector cells and/or a differential capability of premalignant Schwann cells to escape or counteract the cellular immune response. (Cancer Res 2006; 66(9): 4708-14)

Note: J.A.M. Marx is the recipient of a scholarship from the Graduate Program of the Deutsche Forschungsgemeinschaft, Bonn “Pathogenesis of nervous system diseases.”

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tissue of tumor-sensitive BDIX rats as a function of time after N-ethyl-N-nitrosourea exposure (9). Similar amounts of neu-mutant cells were detected in the trigeminal tissue of both strains during the first 100 days after pulse exposure to N-ethyl-N-nitrosourea on postnatal day 1. However, in contrast to the progressive multiplication of neu-mutant cells in BDIX trigeminal nerves during the intermediary phase of carcinogenesis, their numbers gradually decreased in BDIV rats. After 250 days, all BDIX rats had succumbed to their trigeminal tumors, whereas BDIV rats survived devoid of neu mutant cells. Given the fact that the mutant cells were no longer detectable at the brain-nerve junction nor in the more distal part of the nerve, it became clear that premalignant cells could not have differentiated and/or migrated as part of a tissue remodeling process as has been suggested for preneoplastic rat mammary and liver tissue (10). Tumor resistance in the BDIV rat was, thus, considered to be due to mechanisms, including the recognition and removal of premalignant neu-mutant schwannoma precursor cells from trigeminal nerves. It is likely that these cells die through programmed cell death either triggered by tissue intrinsic mechanisms or by host factors, such as the immune system.

To gain insight into the underlying molecular and cellular processes, we have now investigated the potential involvement of a cellular immune response using immunohistochemistry with antibodies against immune cell subpopulations on serial tissue sections of trigeminal nerves prepared at different times after exposure to N-ethyl-N-nitrosourea.

Materials and Methods

Animals and carcinogen exposure. Inbred BDIX and BDIV rats (3) were kept under standard conditions in the animal facility of the Institute of Cell Biology (Cancer Research), University of Duisburg-Essen, Medical School. Rats of both strains received a single s.c. injection of N-ethyl-N-nitrosourea (80 μg/g body weight) 24 hours postnatally (11). Control animals remained without treatment.

Preparation of histologic samples. From postnatal day 40 onwards, groups of three to four rats were sacrificed with CO2 at 10-day intervals. The intracraniatal portions of the trigeminal nerves were dissected out under a stereo operation microscope, snap frozen in liquid nitrogen, and stored at −80°C until cryosectioning (6). In most cases, both nerves were used. Frozen sections (8 μm) were dried and stained with Mayer’s hemalun.

Immunohistochemistry. The primary antibodies used to detect immune effector cells in rat trigeminal nerves were mouse monoclonal antibody (mAb) anti-rat CD18 (clone WT.3; PharMingen, Becton Dickinson, Hamburg, Germany) as a pan-leukocyte marker, mouse mAb anti rat T-cell receptor α/β (clone R73, Serotec, Düsseldorf, Germany) for T cells, mouse mAb anti rat CD4 for T helper cells and macrophages (clone W3/25; Serotec) mouse mAb anti-rat CD8 (clone MRC-OX-8, Serotec) for cytotoxic T lymphocytes (CTLs), as well as mouse mAbs ED1 and ED2 (Serotec) for distinct macrophage subpopulations. Furthermore, mouse mAb anti-CD45 RA (clone OX-33; PharMingen) was used to detect B lymphocytes and mouse mAbs HIS48 (Serotec) and anti-CD161 (clone 10/78; Serotec) for cells of the myeloid lineage and natural killer (NK) cells, respectively. Rat mesenteric lymph nodes served as positive control tissues. Antigen-specific binding was detected with a Biotin-SP-conjugated affinity purified F(ab)2 fragment of rat anti-mouse IgG (H+L; Jackson ImmunoResearch, Dianova, Hamburg, Germany) as secondary antibody followed by avidin-biotin/horseradish peroxidase complex (Dako, Hamburg, Germany). Diaminobenzidine (Sigma FAST DAB, Sigma, Munich, Germany) was used as substrate.

Microscopy and image analysis. Stained sections were first examined under an Olympus stereo-microscope. An automatic image cytometry system (Ahrens ICM, Bargteheide, Germany) was used to determine the numbers of antigen expressing cells per mm². For this purpose, the area of each trigeminal nerve section was operationally divided into two zones: region A, located adjacent to the brain-nerve junction and covering about 350 μm of the nerve’s length and region B, defined as the remaining peripheral portion of the intracranial part of the nerve (see Fig. 1).

Statistical analysis. For each immune cell type count, a four factorial ANOVA was done with the factors “region,” “strain,” “time point,” and “treatment,” including all 2-fold interactions. Because the interaction term of strain by time for CD4 cells was significant at the 10⁻⁴ level, indicating different time effects for BDIV and BDIX rats, it was decided to analyze both strains separately. Because the group sizes were unbalanced, procedure GLM of the program SAS V8.02 for Win NT was used. The Ps presented are not adjusted for multiple testing.

Results

Histomorphologic observations. To assess the involvement of a cellular immune response in the recognition and elimination of premalignant neu-mutant Schwann cells trigeminal nerve sections from N-ethyl-N-nitrosourea–treated rats and from untreated control animals of both strains were stained with mAbs directed against cell surface determinants specific for different subpopulations of cells of the immune system (Fig. 2).

The time points chosen were 10-day intervals beginning on day 40 after N-ethyl-N-nitrosourea exposure to day 250. For each time point, trigeminal nerve sections from three to four N-ethyl-N-nitrosourea–treated animals and two to three controls of both strains were microscopically examined, with special attention to the brain-nerve junction.

From postnatal day 40 onwards, CD18-expressing leukocytes were detected in virtually every section from N-ethyl-N-nitrosourea–treated BDIX and BDIV rats and from control animals. More specifically, CD4⁺ cells were seen at all time points investigated, the majority being large with ramified processes. Most of them proved to be macrophages with few T-cell receptor α/β expressing T helper cells, as shown by staining serial sections of different time points with anti-CD4 mAb and anti-T-cell receptor mAb, respectively (data not shown). Very low numbers of ED1 and ED2 expressing macrophages and of CD8⁺ CTLs were seen in trigeminal nerve sections of N-ethyl-N-nitrosourea–treated BDIX and BDIV rats and controls between postnatal days 40 and 80, with a subsequent marked increase.
As a tendency, the amount of stained immune cells at time points >80 days was considerably higher in the nerves of N-ethyl-N-nitrosourea–treated animals compared with the untreated controls (Fig. 2).

Although single NK cells were occasionally seen (data not shown), neither B lymphocytes nor granulocytes were detected in trigeminal sections of any strain at any time point regardless of N-ethyl-N-nitrosourea treatment.

Figure 2. Trigeminal nerve sections (postnatal days 90-100) from N-ethyl-N-nitrosourea (EtNU)–treated BDIV and BDIX rats and controls stained with antibodies specific for CD18, CD4, CD8, ED1, or ED2.
Quantification of antibody-binding immune cell subpopulations. Immune cell subpopulations invading trigeminal nerves were quantified with the use of an automatic image analysis system. For automated counting of stained cells, trigeminal nerve sections were operationally divided into two regions: region A, encompassing the zone adjacent to the brain-nerve junction, where most premalignant neu/erbB-2 mutant cells reside and region B, representing the more distal part of the intracranial portion of the nerve (Fig. 1). As the area of the trigeminal nerve tissue varies between specimens, cell numbers represent counts/mm².

Compared with untreated controls, markedly elevated amounts of CD18⁺ leukocytes were found in region A of trigeminal nerve sections from N-ethyl-N-nitrosourea–treated rats at all times measured, with a less pronounced increase in region B (Fig. 2, I). Accordingly, counts of CD4-positive macrophages/T helper cells (Fig. 2, II) were constantly increased in trigeminal nerves of N-ethyl-N-nitrosourea–treated rats between days 40 and 250 after the carcinogenic pulse, with cell counts in region B being mostly lower than in region A. CD8-expressing CTLs and ED1⁺ macrophages (Fig. 2, III and IV) were barely detectable in trigeminal nerve sections of N-ethyl-N-nitrosourea–treated and control rats from postnatal day 40 until day 70. From day 80 onwards, the numbers of these cells markedly increased, and a difference between N-ethyl-N-nitrosourea–exposed rats and controls became obvious. The numbers of ED2⁺ macrophages/mm² in the trigeminal nerves of N-ethyl-N-nitrosourea–treated BDIX rats were moderately increased over cell numbers obtained for control animals in regions A and B, whereas this was not seen in BDIV animals (Fig. 2, V). However, for all other cell subpopulations investigated, no gross differences in cell number were observed in trigeminal nerves of N-ethyl-N-nitrosourea–treated susceptible rats.

Statistical analysis. To determine which experimental variables significantly influence immune cell density in trigeminal nerve tissue, a four-factorial ANOVA, including 2-fold interactions, was done for each immune cell subpopulation separately. The time points 40 to 130 days were chosen for this purpose, as later on, full blown tumors were already seen in some of the BDIX rats. The density of CD18⁺, CD4⁺, CD8⁺, and ED1⁺ immune cells residing in trigeminal nerve tissue strongly depends on N-ethyl-N-nitrosourea–treatment (significant at the 10⁻⁴ level; Table 1). The time after N-ethyl-N-nitrosourea exposure influences the density of CD18⁺, CD8⁺, ED1⁺, and ED2⁺ cells reflecting mainly the fact that on days 40 to 70, relatively low cell numbers were found compared with later time points (see Fig. 3). For CD18⁺, CD4⁺, and ED2⁺ cells, the tissue segment investigated [brain-adjacent (A) or peripheral (B)] had a remarkable effect on cell density (Ps between <0.001 and <0.0001). The strain of origin does not seem to affect the immune cell counts considerably. However, the numbers of CD4-expressing cells (for which the interaction term of strain by time was significant at the 10⁻⁴ level) were subject to different time effects in BDIV and BDIX rats.

Additionally, looking at both rat strains separately, we saw a marked effect of the region on CD4⁺ and CD8⁺ cell counts/mm² in BDIV rats, which was not detectable in BDIX animals (Table 2). The numbers of ED2⁺ cells/mm² showed a significant increase as a result of treatment in BDIX rats only.

Discussion

We have previously shown that the mechanisms responsible for resistance towards N-ethyl-N-nitrosourea–induced tumorigenesis in the PNS of BDIV rats include the recognition and elimination of premalignant neu/erbB-2 mutant Schwann precursor cells (9). The potential involvement of a cellular immune response in this process was investigated in the present study. In the normal rat PNS, a small population of macrophages expressing ED1, ED2, CD4, and MHC antigens resides in the endoneurium (12). If the immune system were involved in the resistance mechanisms to N-ethyl-N-nitrosourea–induced rat schwannomagenesis immune effector cells should be present in trigeminal nerve tissue of N-ethyl-N-nitrosourea–treated BDIX rats in quantities exceeding the background values substantially.

It has often been shown that human as well as experimental tumors are infiltrated by various types of immune effector cells predominantly of CD4⁺ T helper cells, CD8⁺ cytotoxic T cells, macrophages, and NK cells (13, 14). A correlation between the extent of invasion of CTLs and the patients’ survival has been shown (e.g., in human colorectal cancer; ref. 15). However, complete remissions were never observed. It has, therefore, been suggested that immunosurveillance might play a critical role in suppressing tumor growth during premalignant states (16). Different from the work done on clinically detected tumors, the present study aimed at elucidating whether and when during early post-initiation stages of chemically induced neuro-oncogenesis a cellular immune

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<th>Table 1. Effects of experimental variables on immune effector cell counts in the trigeminal nerves of BDIV and BDIX rats</th>
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<td><strong>Cell counts</strong></td>
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<td><strong>Treatment (t)¹</strong></td>
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Note: +, \( P < 0.05 \); *, \( P < 0.01 \); ***, \( P < 0.001 \); ***, \( P < 0.0001 \).
Figure 3. Counts of individual immune effector cells/mm² in the brain-adjacent (region A, see left) and peripheral part (region B, see right) of trigeminal nerve sections from N-ethyl-N-nitrosourea–treated BDIV and BDIX rats and control animals as a function of time. Dark curves, values for N-ethyl-N-nitrosourea–treated animals; light curves, values for control animals.
Table 2. Effects of experimental variables on immune effector cell counts calculated separately for trigeminal nerves of BDIV and BDIX rats

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<th>Cell counts</th>
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NOTE: +, \( P < 0.05 \); *, \( P < 0.01 \); **, \( P < 0.001 \); ***, \( P < 0.0001 \).

These cells subsequently acquire fully malignant properties and later on kill the affected animals in the presence of markedly elevated numbers of cytotoxic T cells, T helper cells, and CD4- and ED1-expressing macrophages equaling the numbers recorded in trigeminal nerve tissue of N-ethyl-N-nitrosourea–treated BDIV rats. Surprisingly, the numbers of ED2+ macrophages significantly exceeded the values of BDIX control rats, which was not observed in BDIV rats. It has previously been shown that a high amount of innate immune cells, such as macrophages, mast cells, and neutrophilic granulocytes, in neoplastic tissues correlates with a poor prognosis (19). Innate immune cells can contribute to cancer development by the production of growth factors, cytokines, chemokines, and matrix metalloproteinases, leading to increased cell survival, tissue remodeling, the promotion of angiogenesis, and suppression of the adaptive immune response. As elevated amounts of ED2+ macrophages are solely detected in trigeminal nerves of tumor-sensitive BDIV rats during later stages of tumor development, it has to be considered whether they support malignant progression of initiated Schwann cells.

The numbers of all other immune effector cells did not grossly differ between N-ethyl-N-nitrosourea–treated tumor-sensitive BDIX and resistant BDIV rats. This might mean that the presence of T lymphocytes and ED1+ macrophages is of no functional significance for the elimination of premalignant Schwann precursor cells. Investigations into the resistance mechanisms of Copenhagen rats to the chemical induction of mammary cancer in crosses with athymic nude rats have revealed an equal tumor incidence in nude F2 animals compared with their non-nude littermates (20). The authors concluded that T cells cannot, therefore, be involved in the mammary cancer resistance of Copenhagen rats. However, the about 100-fold increased incidence of N-ethyl-N-nitrosourea–induced CNS tumors in T cell–depleted thymectomized BDIX rats argues against this option for nervous system tumors.4

PNS tumor susceptibility/resistance in the rat might, therefore, either involve functional differences of the cellular immune response or a differential capability of premalignant cells to escape or counteract the immune system. Strain-specific gross functional differences of immune effector cells are less likely, because an

response occurs and whether a differential efficacy of this response might be reflected by tumor susceptibility or resistance, respectively. The model system used here is well suited for this purpose as (a) the exact localization of the malignant tumors developing later on is known from the very beginning and (b) premalignant cells can be unequivocally identified and localized by using the neu/erbB-2 mutation as a molecular tag and by histologic criteria.

A marked inflammatory reaction was observed in the trigeminal nerves of N-ethyl-N-nitrosourea–treated tumor-sensitive BDIX animals and tumor-resistant BDIV rats beginning as early as postnatal day 40 before full-blown malignant schwannoma cells are present (6). Thus, early alterations in future cancer cells may be able to provoke an immune response supporting the hypothesis of cancer immunosurveillance (17, 18). It remains to be shown whether the neu/erbB-2 mutation in premalignant Schwann precursor cells is the target for the cellular immune response observed. Compared with untreated controls, an increase in the counts of CD18+ leukocytes was found predominantly in region A of trigeminal nerve tissue from N-ethyl-N-nitrosourea–treated rats of both strains where most of the initiated neu-mutant Schwann precursor cells reside. More specifically, increased numbers of CD4-positive macrophages and T helper cells were recorded at all time points, whereas elevated quantities of CD8-expressing CTLs and ED1+ macrophages were detected from day 80 following exposure to N-ethyl-N-nitrosourea. The numbers of ED2+ macrophages were significantly elevated in N-ethyl-N-nitrosourea–treated BDIX rats only, starting on day 80, too. In BDIV control rats, this cell population was larger than in untreated BDIX rats at all time points measured but did not increase as a consequence of N-ethyl-N-nitrosourea treatment.

Considerable fluctuations of immune effector cell numbers at successive time points were observed in the curves representing immune cell counts as a function of time (Fig. 2) mainly of BDIX rats. They may reflect interaction of premalignant and malignant neu/erbB-2–mutated Schwann cells with immune cells, which could result in repeated immune cell reductions with subsequent increases.

Premalignant neu/erbB-2 mutant Schwann cells gradually disappear in BDIV rats beginning on day 80 after N-ethyl-N-nitrosourea exposure, just at the time when significantly elevated numbers of CTLs and macrophages are present. At the same time, neu/erbB-2 mutant Schwann cells rapidly multiply in the BDIX rat.

impairment of the cellular immune response should also affect infection immunity or graft rejection, which was never observed thus far. Rather, a different genetic background might lead to a differential type of interaction between premalignant cells and immune effector cells causing a T lymphocyte–mediated elimination of premalignant cells in the BDIV rat while tumor tolerating, if not promoting conditions may be created in BDIV rats possibly due to the invasion of ED2+ macrophages. In a mouse model of skin carcinogenesis, infiltration of bone marrow–derived cells has previously been shown to be critical for distinct processes of epithelial carcinogenesis as regulation of oncogene-induced keratinocyte hyperproliferation, progression to invasive cancer, and end-stage malignant grade. These effects were mediated by the secretion of matrix metalloproteinase 9 (MMP9) by innate immune cells (21). MMP9 is involved in various processes during the development of human and experimental cancers (22), including immune escape mechanisms as the suppression of T-lymphocyte proliferation. Future investigation will show whether MMP9 plays a role in N-ethyl-N-nitrosourea–induced PNS carcinogenesis.

Tumor cells, too exert a large spectrum of defense mechanisms against T-cell dependent antitumor immunity (18, 23). Transplanted malignant BDIV rat schwannomas have been shown to secrete transforming growth factor β, thereby inhibiting T-cell proliferation (24). However, T-cell targeting defense mechanisms are likely to be just part of an immunosuppressive network created by interactions of premalignant cells with host stromal cells that promote tumor growth and protect the tumor cells from immune attack (23). Mediators of inflammation such as tumor necrosis factor α (TNFα) and nuclear factor-κB (NF-κB) play a key role in these processes (14). TNFα−/− knockout mice are resistant to chemically induced skin carcinogenesis (25). TNFα does not influence the initiation phase of skin carcinogenesis, as the initiating H ras mutation also occurred in its absence but proved to be a critical mediator of tumor promotion just as NF-κB in inflammation induced hepatic cancer (26, 27). As the resistance mechanism against N-ethyl-N-nitrosourea–induced PNS tumors comes into effect during later stages of carcinogenesis (9), relevant candidate genes influencing tumor risk, should exert their actions during the post-initiation period. This and the persistent immune response observed in the trigeminal nerves of N-ethyl-N-nitrosourea–treated animals of both strains with differential results in terms of tumor rejection invites the idea that in N-ethyl-N-nitrosourea–induced PNS carcinogenesis genes encoding immune modifiers might be crucial for cancer susceptibility and resistance.

Acknowledgments

Received 10/19/2005; revised 2/14/2006; accepted 2/28/2006.

Grant support: Wilhelm-Sander-Stiftung, Munich, Germany grant 94.009 (A. Kindler-Röhborn and M.F. Rajewsky).

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We thank Claudia Lechleiter, Thomas Haberland, and Michael Opp [Institute of Cell Biology (Cancer Research), University of Düsseldorf-Essen Medical School] for expert assistance with animal husbandry and Dr. Dirk Wedekind [Institute for Laboratory Animal Breeding, Medical School, Hannover, Germany] and Prof. Thomas Herrmann [Institute of Virology and Immunobiology, Würzburg, Germany] for critically reading the article and for stimulating discussions.

References

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