

Inhibition of Methylcholanthrene-induced Carcinogenesis by an Interferon γ Receptor-dependent Foreign Body Reaction

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Abstract

The foreign body reaction is one of the oldest host defense mechanisms against tissue damage which involves inflammation, scarring, and encapsulation. The chemical carcinogen methylcholanthrene (MCA) induces fibrosarcoma and tissue damage in parallel at the injection site. Tumor development induced by MCA but not due to p53-deficiency is increased in interferon- γ receptor (IFN- γ R)-deficient mice. In the absence of IFN- γ R, MCA diffusion and DNA damage of surrounding cells is increased. Locally produced IFN- γ induces the formation of a fibrotic capsule. Encapsulated MCA can persist virtually life-long in mice without inducing tumors. Together, the foreign body reaction against MCA prevents malignant transformation, probably by reducing DNA damage. This mechanism is more efficient in the presence of IFN- γ R. Our results indicate that inflammation and scarring, both suspected to contribute to malignancy, prevent cancer in certain situations.

Key words: inflammation • tissue damage • tissue repair • encapsulation • immune surveillance

Introduction

Chemical carcinogens such as polycyclic aromatic hydrocarbons (PAH)* are contained in environmental pollutants and appear to contribute to cancer in man (1). One type of PAH is 3-methylcholanthrene (MCA) which has been widely used in mice to analyze chemical carcinogenesis (2). MCA usually induces tumors at the site of injection, for unknown reasons often fibrosarcomas. Malignant transformation by MCA results from the stepwise accumulation of mutations and selection of cells with the right mutations, e.g., in protooncogenes like ras or tumor suppressor genes like p53 (3, 4). MCA induces mutations in a random fashion and many mutations might interfere with cell integrity. Thus, MCA should induce tissue damage and malignant transformation in parallel (5).

The repair process that follows injury in a variety of tissues resembles the process of wound healing of the skin (6). Wound repair in adult skin involves a series of overlapping and highly orchestrated events involving inflammation, reepithelialization, granulation tissue formation, and, finally, scar formation (7). The inflammatory response to tissue

damage is characterized by the rapid influx of neutrophils followed by the accumulation of macrophages. In the later phases, fibroblasts become the dominant cell type. They produce and deposit extracellular matrix, finally leading to fibrosis or scar formation (6, 7).

Inflammation has been suspected to contribute to cancer for a long time (for a review, see reference 8). Based on observations that carcinomas appear around the wound of skin pretreated with carcinogen (9–11), the initiation/promotion model of carcinogenesis has received much attention. Sub-threshold amounts of PAH induce irreversible cellular changes, probably by inducing first mutation(s). If the same site is treated even after a long time with reagents like TPA (12-O-tetradecanoylphorbol-13-acetate), which are not carcinogenic but induce inflammation, tumors can develop (12). Tumor-promoting effects of inflammation have usually been associated with late stages such as promotion or progression during chemical carcinogenesis (13). More recently, this has been shown during tumor development in oncogene-transgenic mice or tumor suppressor gene-deficient mice with defined inflammatory defects (14–16).

The foreign body reaction is characterized by encapsulation of foreign material. Phylogenetically, it is one of the oldest defense mechanisms, predating adaptive immunity. Encapsulation is a major defense mechanism in insects and molluscs, e.g., if phagocytosis against larger parasites fails (17–19). Even the phylogenetically old porifera can form a

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*Abbreviations used in this paper: MCA, methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

barrier by deposition of a fibrous wall at the interphase to an allograft (20). Therefore, the foreign body reaction appears to be the most common mechanism in multicellular organisms to keep tissue integrity. In man, it is commonly associated with a pathological situation, typically described as fibrous tissue capsule or scar surrounding foreign material like talc crystals as remnants of a surgeon's glove, artificial implants (21), or most dramatically upon exposure to asbestos (22).

IFN- γ is produced by several cell types upon activation (e.g., T, B, NK cells, macrophages, and mast cells; references 23–27), but also appears to be constitutively present at basal levels in nontreated mice (28). The pleiotropic activities of IFN- γ are compatible with the ubiquitous expression of the IFN- γ receptor (IFN- γ R). The immunoregulatory role of IFN- γ for T cell subsets, antigen processing, or MHC expression is well established (23). However, T cells in IFN- γ R^{-/-} mice develop and produce IFN- γ normally (29) and can induce rejection of transplanted tumors in IFN- γ R^{+/+} mice (30). Effects of IFN- γ on function of mesenchymal cells are less clear. It was shown that IFN- γ downregulated fibroblast proliferation and connective tissue production (6). In contrast, recent *in vivo* experiments showed that IFN- γ directly caused growth factor-induced vascular smooth muscle cell mitogenesis and arteriosclerosis which is associated with accumulation of extracellular matrix (31). We show here that chemical carcinogenesis by MCA is inhibited in an IFN- γ R-dependent fashion. Since we had no indications for T cell involvement and the mechanism appeared to be different from T cell-mediated tumor transplantation immunity, we analyzed the local reaction to MCA in the primary host. During the tissue repair response to MCA-induced damage MCA is encapsulated and persists long-term within microscopic scars in tumor-free mice.

Materials and Methods

Mice. IFN- γ R^{-/-} mice, generated as inbred 129/Sv/Ev line, and congenic IFN- γ R^{+/+} control mice were provided by M. Aguet (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland; reference 29). IFN- γ R^{+/-} mice were generated by breeding IFN- γ R^{-/-} with IFN- γ R^{+/+} mice. For most experiments, IFN- γ R^{+/-} and IFN- γ R^{-/-} mice were paired to obtain IFN- γ R-deficient and IFN- γ R-competent control littermates. BALB/c, BALB/c nu/nu mice, and mice homozygous for the p53 tumor-suppressor gene (p53^{-/-} mice; reference 32) with a mixed genetic background of 129/Sv/Ev and C57BL/6 were obtained from Bomholtgaard Breeding Facilities, Denmark. The p53 and IFN- γ R double knockout mice were generated by breeding a single p53^{-/-} male with several IFN- γ R^{-/-} female mice. F1 mice were intercrossed to obtain p53^{+/-}IFN- γ R^{+/-}, p53^{+/-}IFN- γ R^{-/-}, p53^{-/-}IFN- γ R^{+/-}, and p53^{-/-}IFN- γ R^{-/-} mice. Genomic typing of mice was performed by PCR as described previously (29, 32). All mice except for nude mice were bred in the animal facility of the Max-Delbrueck-Center of Molecular Medicine under germ-poor conditions. They were regularly tested and found free for a panel of pathogens (available upon request) throughout the study.

Tumor Induction. MCA-induced tumorigenesis in IFN- γ R-competent and -deficient mice was analyzed beginning in 1994 in three subsequent experiments. Mice were injected intramuscularly in the left hind leg with different amounts of MCA (Sigma-Aldrich) suspended in 0.1 ml of sesame oil. Tumor development was observed 2–3 times weekly for 1.5–2 y. Mice with a tumor >10 mm in diameter were counted as tumor positive. Spontaneous tumor development in the absence of one or both p53 alleles was monitored 1–3 times weekly. Mice were killed when they had an overt mass of \geq 15 mm or appeared moribund. The diagnosis of tumors was done by macroscopic analysis of different organs, microscopy of peripheral blood smears, and histological analysis.

Tumor Cell Lines. MCA-induced primary tumors from IFN- γ R^{+/+} and IFN- γ R^{-/-} mice (H-2^b) were surgically excised and passaged as tumor fragment (4 × 4 × 4 mm) in BALB/c nu/nu mice (H-2^d) to minimize the contamination of stromal cells from primary tumor-bearing mice during flow cytometric analysis. The passaged tumors were minced, trypsin-treated for 5 min at 37°C, and maintained by culture in RPMI 1640 medium supplemented with 10% FCS. J558-IFN- γ and J558-LT cells have been described previously (33, 34). 5 × 10⁵ of the cytokine gene transfected cells produce 10 ng/ml IFN- γ as measured by ELISA or 330 U/ml LT in 24 h as measured by L929 cytolytic assay, respectively.

Flow Cytometric Analysis. Tumor cells prepared as above and cultured in the third passage were stained with FITC-conjugated mAbs against H-2D^b (HK95), H-2K^b (AF6-88.5), I-a^b (06044D), or isotype-matched control mAb, rat IgG_{2b} (11034C) and rat IgG_{2a} (11024C; BD PharMingen). To ascertain the success of bone marrow reconstitution of IFN- γ R^{+/+} and IFN- γ R^{-/-} mice, 3 mo after bone marrow transplantation, peripheral blood cells were stained with mAbs specific for murine B220, CD4, CD8, and GR-1 in combination with anti-IFN- γ R mAb (GR-20; BD PharMingen). As control isotype-matched rat Ig was used. Three mice from each group were analyzed. All samples were analyzed with an Epics-XL flow cytometer (Beckman Coulter).

Tumor Fragment Transplantation. To avoid phenotypic changes during *in vitro* culture, instead of cultured tumor cells, tumor fragments were used to analyze tumor growth. MCA-induced tumors from primary tumor-bearing mice were surgically isolated and cut into pieces of 4 × 4 × 4 mm. The tumor fragments were then subcutaneously transplanted onto IFN- γ R^{+/+} mice in the left side of abdomen. The growth of tumors was monitored every 2–3 d. Together, five IFN- γ R^{+/+} and five IFN- γ R^{-/-} tumors were analyzed.

Bone Marrow Chimera. Freshly prepared bone marrow cells of 6–8-wk-old IFN- γ R^{+/+} or IFN- γ R^{-/-} mice were harvested from femurs and tibiae and 10⁷ cells were injected intravenously into lethally irradiated (10 Gy) age- and sex-matched mice. The following groups were included: IFN- γ R^{+/+} → IFN- γ R^{+/+}; IFN- γ R^{+/+} → IFN- γ R^{-/-}; IFN- γ R^{-/-} → IFN- γ R^{-/-}; and IFN- γ R^{-/-} → IFN- γ R^{+/+}. Successful reconstitution of the hematopoietic system was determined by flow cytometric analysis of peripheral blood mononuclear cells for IFN- γ R expression. Additionally, chimerism of mice was confirmed by PCR analysis of the IFN- γ R gene using tail DNA that contained genomic DNA from both nonhematopoietic and hematopoietic cells.

Histology. Mice were intramuscularly injected with MCA in the left hind leg and after the indicated periods, tissue from the injection site was isolated and cryostat sections were prepared. MCA was detected by light microscopy on Hematoxylin-stained sections and by fluorescent microscopy. We noticed that acetone fixation could lead to loss of MCA from the tissue section. For

immunohistological analysis, alkaline phosphatase staining was done as described previously (35). The mAbs used were anti-CD4 (RM4-4), anti-CD8 (53-6.7), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5) (BD PharMingen), anti-fibroblast/extracellular matrix (ER-TR7) (BMA, Augst, Swiss), anticollagen I-V (2150-2206) (Biotrend), and isotype-matched control mAbs. Alkaline phosphatase-conjugated goat anti-rat IgG and rabbit anti-goat IgG were purchased from Jackson Immunoresearch Laboratories. To analyze the effect of local IFN- γ production on extracellular matrix deposition, 5×10^6 J558-IFN- γ and as control, J558-LT cells were injected subcutaneously into nude mice and tumor tissue was obtained after 3-4 wk. Collagen was visualized by Verhoeff-van Gieson staining. To detect DNA damage on MCA-containing sections, an in situ cell death detection kit was used according to the manufacturer's instructions (Boehringer Mannheim). The breaks of single- or double-stranded DNA are visualized by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

Diffusion of MCA/Oil. Abdominal skin of mice was shaved or depilated with Veet Sensitive Plus Enthaaungs-Creme (Reckitt Benckiser) and 0.8 mg of MCA in 0.2 ml sesame oil was subcutaneously injected into the depilated area, causing a bubble-like swelling. Mice were weekly observed for persistence of the bubble (>2 mm in diameter). In parallel, histological analysis confirmed that the swelling was always due to the local presence of MCA/oil.

Results

Low Incidence of MCA-induced Tumors in IFN- γ R-competent Compared with IFN- γ R-deficient Mice without Evidence of Immune Selection. IFN- γ R^{+/+}, IFN- γ R^{+/-}, and IFN- γ R^{-/-} mice were injected intramuscularly with 0.8 mg MCA and tumor development was observed for 80 wk. In repeated experiments, IFN- γ R^{-/-} mice developed tumors earlier and with higher frequency compared with IFN- γ R^{+/+} or IFN- γ R^{+/-} mice. For example, all male IFN- γ R^{-/-} (35/35) but only 44% (16/36) IFN- γ R^{+/-} mice had developed tumors at week 38 (Fig. 1 a). At week 80, 31% of the male IFN- γ R^{+/-} mice remained tumor free. Similar results were observed with female mice (Fig. 1 a). IFN- γ R^{+/+} and IFN- γ R^{+/-} mice developed tumors with similar frequency and kinetics (Fig. 1 b). If mice were treated with lower amounts of MCA, namely, 0.3 mg (Fig. 1 c) or 0.1 mg (Fig. 1 d), the difference in tumor incidence between IFN- γ R^{+/-} and IFN- γ R^{-/-} group was again significant. To ask whether the decreased tumor development in IFN- γ R-competent mice was associated with signs of immune selection, tumors of IFN- γ R^{+/+} and IFN- γ R^{-/-} mice were analyzed for some indicative cell surface mark-

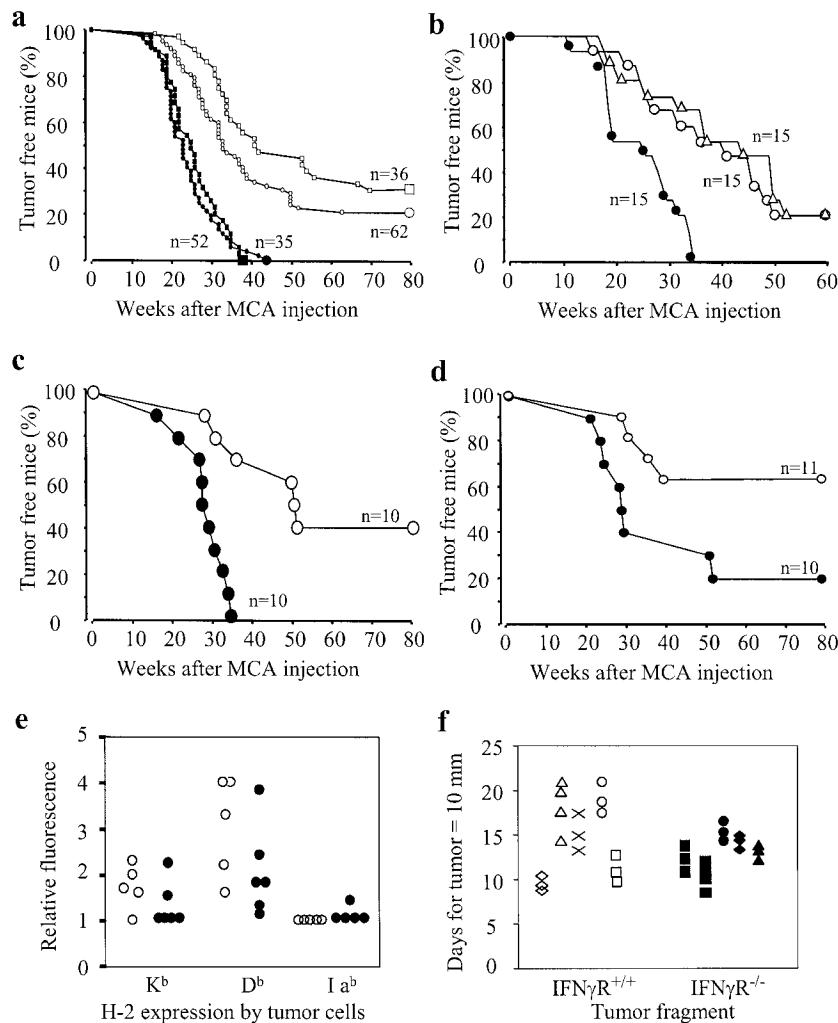


Figure 1. Inhibition of MCA-induced tumorigenesis in 129Sv/Ev IFN- γ R^{+/+} and IFN- γ R^{+/-} compared with IFN- γ R^{-/-} mice without evidence of immune selection. (a-d) Tumor incidence. 6-10-wk-old IFN- γ R^{+/-} (white symbols) and IFN- γ R^{-/-} (black symbols) male (□ and ●) or female mice (○ and △) were intramuscularly injected with (a and b) 0.8 mg, (c) 0.3 mg, and (d) 0.1 mg MCA, and tumor development was monitored. Mice with a tumor of ≥ 10 mm were recorded as tumor positive. In b, tumor incidence in IFN- γ R^{+/+} female mice (△) was also shown. The number of mice/group is indicated. In a, one out of three experiments with similar results is shown. (e) Expression of MHC-molecules on MCA-induced IFN- γ R^{+/+} (white) and IFN- γ R^{-/-} (black) tumor cells. Cells were stained with FITC-conjugated mAbs against H-2K^b, H-2D^b, and I-a^b. Shown are folds of mean channel fluorescence above background. Each symbol represents the staining of one primary cell culture. (f) Similar growth of IFN- γ R^{+/+} and IFN- γ R^{-/-} tumors in wild-type mice. Fragments of MCA-induced tumors (4 × 4 × 4 mm) were transplanted subcutaneously onto IFN- γ R^{+/+} mice. Tumor size was monitored every 2-3 d. Shown are days required for a tumor to grow to the size of 10 mm. Each symbol represents the growth of one primary tumor grafted onto 3-4 recipient mice. Five IFN- γ R^{+/+} (white) and five IFN- γ R^{-/-} tumors (black) were analyzed.

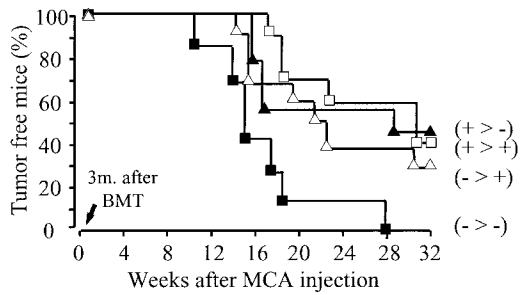


Figure 2. IFN- γ responsiveness of either bone marrow-derived or nonbone marrow-derived cells is sufficient for inhibition of MCA-induced tumorigenesis. IFN- γ ^{+/+} (white) and IFN- γ ^{-/-} mice (black) were lethally irradiated and reconstituted with bone marrow cells from mice with the same (□ and ■) or different genotype (△ and ▲). 3 mo after reconstitution mice were intramuscularly injected with 0.8 mg MCA in the left hind leg and tumor development was monitored. Each group contained 7–11 mice. Shown is the experiment with male mice. Similar results were obtained when female mice were analyzed.

ers. Overall, expression of MHC class I (D^b and K^b), MHC class II (Fig. 1 e), vascular cell adhesion molecule 1, intracellular adhesion molecule 1, B7.1, and B7.2 molecules (data not shown) was similar in tumors of both groups. IFN- γ ^{+/+} and IFN- γ ^{-/-} tumor fragments were transplanted onto IFN- γ ^{+/+} recipients. As can be seen in Fig. 1 f, tumors of both groups grew with similar kinetics. Therefore, more efficient control of MCA-induced car-

cinogenesis in the presence of IFN- γ R occurred without obvious indications of immune selection.

IFN- γ R Expression by Either Bone Marrow- or Nonbone Marrow-derived Cells Is Sufficient to Inhibit MCA-induced Tumor Development. Previously, we showed that T cell-mediated tumor transplantation immunity required IFN- γ R expression by nonhematopoietic but not hematopoietic or tumor cells (30). To ask whether control of chemical carcinogenesis involved similar mechanisms, bone marrow chimeras were generated and 3 mo after reconstitution challenged with MCA. Comparison of tumor development between IFN- γ ^{+/+} → IFN- γ ^{+/+} and IFN- γ ^{-/-} → IFN- γ ^{-/-} bone marrow chimeric mice confirmed that IFN- γ responsiveness decreased tumor incidence (Fig. 2). Surprisingly, both IFN- γ ^{-/-} → IFN- γ ^{+/+} and IFN- γ ^{+/+} → IFN- γ ^{-/-} groups of mice showed decreased tumor development, similar to the IFN- γ ^{+/+} → IFN- γ ^{+/+} mice. Thus, IFN- γ R expression by either bone marrow- or nonbone marrow-derived cells was able to inhibit MCA-induced carcinogenesis. This suggested that control of chemical carcinogenesis is mechanistically different from that of transplanted tumors.

Tumor Development Due to p53 Deficiency Is Not Changed in IFN- γ ^{-/-} Compared with IFN- γ ^{+/+} Mice. To analyze whether the IFN- γ -mediated control mechanism is also operative against spontaneous tumor development, we generated mice defective in both IFN- γ R and p53 genes. p53-deficient mice are healthy at birth and invariably de-

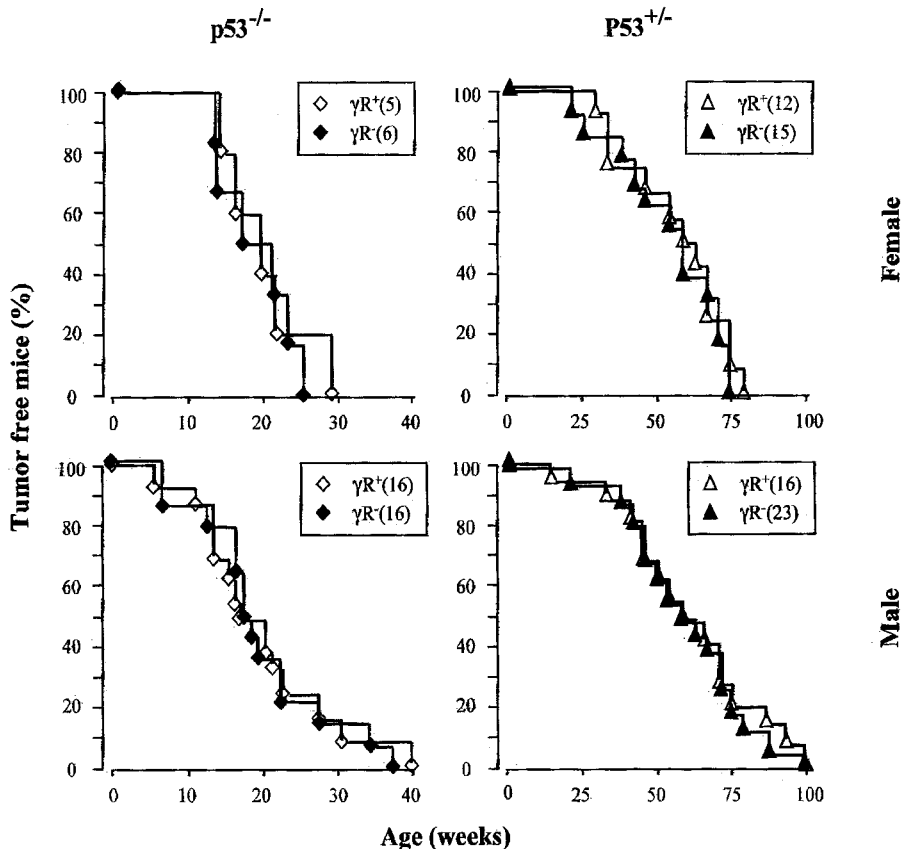


Figure 3. Tumor development due to p53-deficiency is not changed in IFN- γ ^{+/+} compared with IFN- γ ^{-/-} mice. IFN- γ ^{+/+}p53^{-/-} (◇), IFN- γ ^{-/-}p53^{-/-} (◆), IFN- γ ^{+/+}p53^{+/-} (△), and IFN- γ ^{-/-}p53^{+/-} mice (▲) were monitored for tumor development. Results from female (top) and male mice (bottom) are plotted separately. Mice, which were moribund or had a solid tumor of 15 mm, were recorded as tumor positive. No significant difference in tumor types between IFN- γ ^{+/+} and IFN- γ ^{-/-} mice was detected. Number of mice per group is given in parenthesis.

velop tumors (32). Importantly, malignant transformation by p53 deficiency occurs in a silent way as opposed to MCA-induced carcinogenesis, which is accompanied by tissue damage (see below). Fig. 3 shows tumor development in $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ mice with one or both defective p53 alleles. In either case, no difference in tumor development was observed between $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ mice. For example, 50% male $IFN-\gamma R^{+/-}/p53^{+/-}$ mice developed a tumor at week 59 and 100% at week 98. $IFN-\gamma R^{-/-}/p53^{+/-}$ mice developed a tumor at week 60 to 50% and week 97 to 100%, respectively. $IFN-\gamma R^{+/-}/p53^{-/-}$ and $IFN-\gamma R^{-/-}/p53^{-/-}$ mice also developed tumors with similar kinetics. There were no significant differences in the tumor spectrum between $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ mice (data not shown). Thus, we could not detect an $IFN-\gamma R$ -mediated mechanism that inhibited spontaneous tumor development.

Tissue Repair Response and Fibrosis at the Site of MCA.

The results so far led us to analyze the local reaction to MCA. Sections of MCA-injected tissues of $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ mice were analyzed for the presence of MCA at different time points after injection (each 3–5 mice at weeks 3, 4, 6, 10, and 20). Regardless of the time after injection and the mouse genotype, MCA was always detected by light microscopy as crystalline structures within the muscle tissue (Fig. 4 a and c). To confirm that these structures were indeed MCA, we made use of the fact that MCA is a polycyclic aromatic and can become visible on tissue sections through auto-fluorescence (Fig. 4 b). Additionally, MCA was associated with local tissue damage. Consecutive tissue sections containing MCA from the above-mentioned mice were analyzed for the presence of cells positive for Mac1, Gr1, CD4, CD8, and γ/δ T cell receptor. γ/δ^+ (data not shown) and $CD8^+$ T cells were

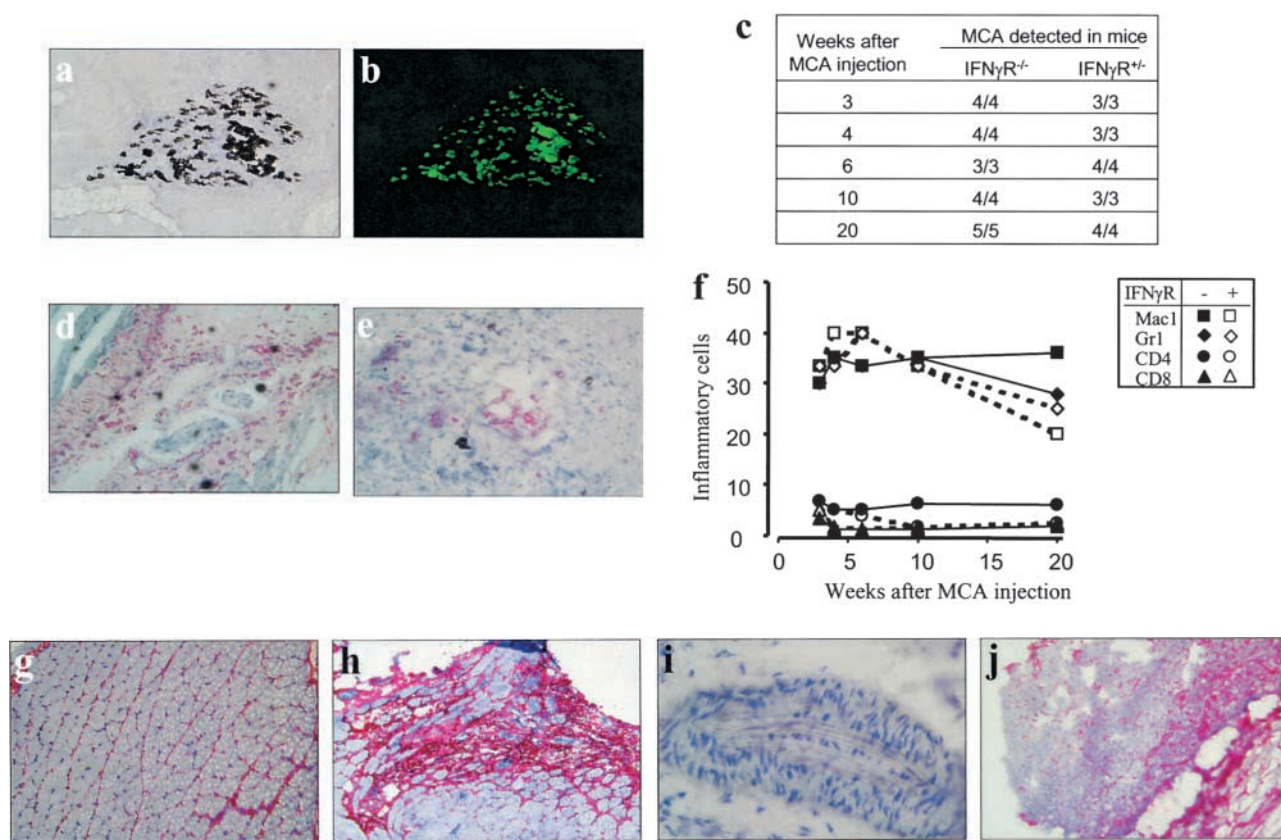


Figure 4. Tissue repair response and fibrosis at the site of MCA. $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ mice were injected intramuscularly with 0.8 mg MCA and tissue sections were prepared 3, 4, 6, 10, and 20 wk after injection (3–5 mice per time point and mouse strain). (a) MCA detected under light and (b) fluorescence microscope. (c) shows numbers of mice in which MCA was detected per numbers of total mice examined at different time points. (d–f) Inflammation at the site of MCA. Consecutive sections of the MCA-containing sections in c were confirmed to also contain MCA and then stained with various mAbs. Since inflammation between $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ was similar, one example for (d) anti-Mac1 and (e) anti-Gr1 staining of tissue sections prepared 10 wk after MCA injection is shown. (f) Summary of the immunohistological staining for Mac1, Gr1, CD4, and CD8 positive cells of the $IFN-\gamma R^{+/-}$ (white) and $IFN-\gamma R^{-/-}$ mice (black) shown in c. Shown are mean numbers of the indicated inflammatory cells surrounding MCA in one optical field with 100 \times magnification. (g–i) Fibroblast accumulation and fibrosis at the MCA-injection site. (g) mAb ER-TR7 staining of a muscle tissue section of a control mouse not injected with MCA and (h) a tissue section with MCA prepared 10 wk after MCA-injection. (i) Hematoxylin staining of a tissue section prepared 20 wk after MCA injection showing fibroblasts closely surrounding a piece of MCA. Fibroblast accumulation at the site of MCA was similarly observed in $IFN-\gamma R^{+/-}$ and $IFN-\gamma R^{-/-}$ mice and at the different time points. (j) Detection of microscopic fibrosarcoma near MCA. 20 wk after MCA injection an early fibrosarcoma with reduced ER-TR7 expression was detected microscopically in two of the $IFN-\gamma R^{-/-}$ mice. Original magnifications: $\times 400$ (i) and $\times 100$ (others).

only occasionally detected at the site of MCA in both groups of mice. CD4⁺ T cells appeared to be more abundant and occurred in similar amounts in IFN- γ R^{+/-} and IFN- γ R^{-/-} mice. Large numbers of Mac1⁺ (Fig. 4 d) and Gr1⁺ cells (Fig. 4 e) were detected in close vicinity to MCA. This was similar at the different time points and between IFN- γ R^{+/-} and IFN- γ R^{-/-} mice, even though we cannot exclude subtle differences in the strength of the inflammatory reaction. The cumulative data of the immunohistochemical analysis are summarized in Fig. 4 f.

Considering that MCA usually induces fibrosarcomas at the injection site and that fibroblasts accumulate at sites of tissue damage, MCA-containing tissue sections were stained with an antibody specific for fibroblasts and extracellular matrix (mAb ER-TR7). Staining of normal muscle tissue sections showed an ordered net-like structure of fibroblasts closely surrounding muscle cells (Fig. 4 g). At the site of MCA, a dramatic accumulation of fibroblasts was detected (Fig. 4 h and i). This was observed similarly in IFN- γ R^{+/-} and IFN- γ R^{-/-} mice and also at the different time points after MCA injection. Thus, fibroblasts might be directly exposed to the mutagenic activity of MCA. In fact, in two out of five IFN- γ R^{-/-} mice analyzed at week 20 after MCA treatment tumors were detected which had strongly reduced expression of the fibroblast marker (Fig. 4 j). It is known that tumors derived from fibroblasts often downregulate cell type-specific markers (36). Together, the local reaction at the site of MCA resembles a tissue repair response, similarly in IFN- γ R^{+/-} and IFN- γ R^{-/-} mice.

Local IFN- γ Production Induces a Collagenous Capsule. Because control of MCA-induced carcinogenesis required

IFN- γ responsiveness, IFN- γ should be locally available. To analyze the effects of local IFN- γ production on the surrounding tissue and extracellular matrix formation, we used the tumor line J558L transfected to secrete IFN- γ (J558-IFN- γ). We knew that tumor growth of these cells is strongly delayed in nude mice due to IFN- γ secretion (33). Sections of J558-IFN- γ tumors grown in nude mice showed that a collagenous capsule clearly separated the whole tumor from neighboring tissue (Fig. 5 a and c). To demonstrate that the formation of extracellular matrix was specific for IFN- γ , we used as a control the same tumor transfected to secrete lymphotoxin (J558-LT; reference 34). Both tumor cell lines grew with similar kinetics in nude mice (data not shown). No collagenous capsule but only scattered collagen fibers could be detected for the J558-LT tumor (Fig. 5 b and d). Therefore, the local IFN- γ production led to encapsulation of transplanted tumor cells.

Inhibition of MCA Diffusion in IFN- γ R^{+/-} Compared with IFN- γ R^{-/-} Mice. Next, a possible relevance of the IFN- γ -induced collagenous capsule for decreased MCA-induced carcinogenesis in IFN- γ R^{+/-} compared with IFN- γ R^{-/-} mice was analyzed. MCA is usually injected as a viscous sesame oil emulsion that diffuses slowly from the injection site. To monitor this diffusion process, MCA/oil emulsion was subcutaneously injected into IFN- γ R^{+/-} and IFN- γ R^{-/-} mice and the kinetics of diffusion was determined. MCA injected subcutaneously or intramuscularly induces tumors with a similar kinetics. In the majority of IFN- γ R^{+/-} mice, swelling caused by the MCA/oil emulsion was visible during a 7-wk period of observation (Fig. 6 a). In contrast, beginning at week 2, a macroscopically visible

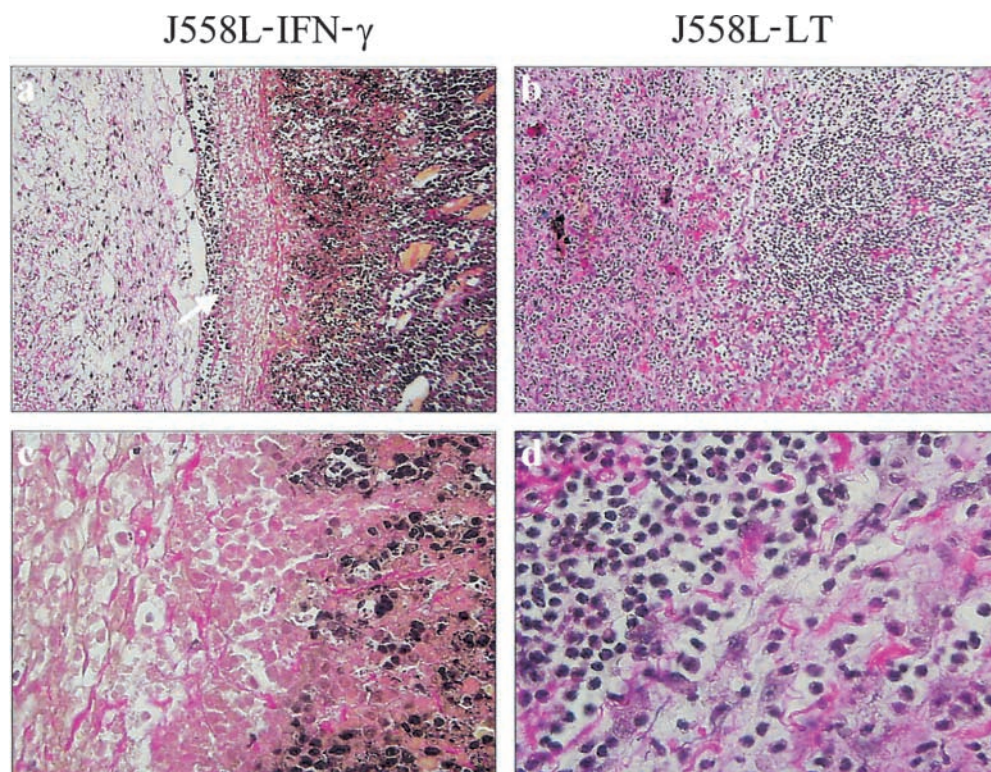


Figure 5. Local production of IFN- γ induces a collagenous capsule. Nude mice were subcutaneously injected with 5×10^6 (a and c) J558-IFN- γ or (b and d) J558-LT tumor cells. 3–4 wk later, when tumors had grown to a size of ~ 10 mm in diameter, paraffin sections were prepared and stained for collagen by the Verhoeff-van Gieson method. The arrow indicates a wall-like collagenous capsule separating normal and tumor tissue. Original magnification: (a and b) $\times 100$; (c and d) $\times 400$. Results are representative for tumor sections of 3–5 mice per group.

swelling disappeared in most of the $\text{IFN-}\gamma\text{R}^{-/-}$ mice indicating that $\text{IFN-}\gamma$ responsiveness causes inhibition of the MCA/oil emulsion to diffuse from the injection site. Immunohistochemical analysis showed a similar accumulation of Mac1^+ and Gr1^+ cells at the injection site as seen for MCA in the muscle (data not shown).

Decreased Tissue Damage at the Site of MCA in $\text{IFN-}\gamma\text{R}^{+/-}$ Compared with $\text{IFN-}\gamma\text{R}^{-/-}$ Mice. MCA induces random mutations. One can expect that many of the mutations do not create a growth advantage and even decrease cell survival. Probably, only in a minority of cases mutations cause a growth advantage and finally malignant transformation. Therefore, cell death and malignant transformation should happen in parallel. As a measurement for exposure of nearby cells to the mutagenic activity of MCA we used the TUNEL-technique which detects DNA-breaks. Tissue sections of $\text{IFN-}\gamma\text{R}^{+/-}$ and $\text{IFN-}\gamma\text{R}^{-/-}$ mice were analyzed at weeks 3, 4, 6, 10, and 20 after MCA injection (the mice which are shown in Fig. 4 c).

An example of TUNEL-staining at the site of MCA 10 wk after injection is shown for $\text{IFN-}\gamma\text{R}^{+/-}$ and $\text{IFN-}\gamma\text{R}^{-/-}$ mice (Fig. 6 b and c). Significantly more DNA breaks were detected in $\text{IFN-}\gamma\text{R}^{-/-}$ compared with $\text{IFN-}\gamma\text{R}^{+/-}$ mice. TUNEL staining always occurred in close vicinity to MCA. The cumulative data of all stainings are expressed as relative index for DNA damage (Fig. 6 d). At all time points, less DNA breaks were detected in $\text{IFN-}\gamma\text{R}^{+/-}$ compared with $\text{IFN-}\gamma\text{R}^{-/-}$ mice. In $\text{IFN-}\gamma\text{R}^{+/-}$ mice, DNA breaks were decreased but not absent which is compatible with the fact that only a part of the mice remained long-term tumor free.

Encapsulated MCA Persists in Long-Term Tumor-free Mice. Taking the above experiments together, the question arose whether encapsulated MCA could be identified in long-term tumor free mice. To exclude mouse strain-specific effects (129/Sv/Ev are more resistant to MCA-induced carcinogenesis compared with BALB/c mice), decreasing amounts of MCA were injected into BALB/c mice anti-

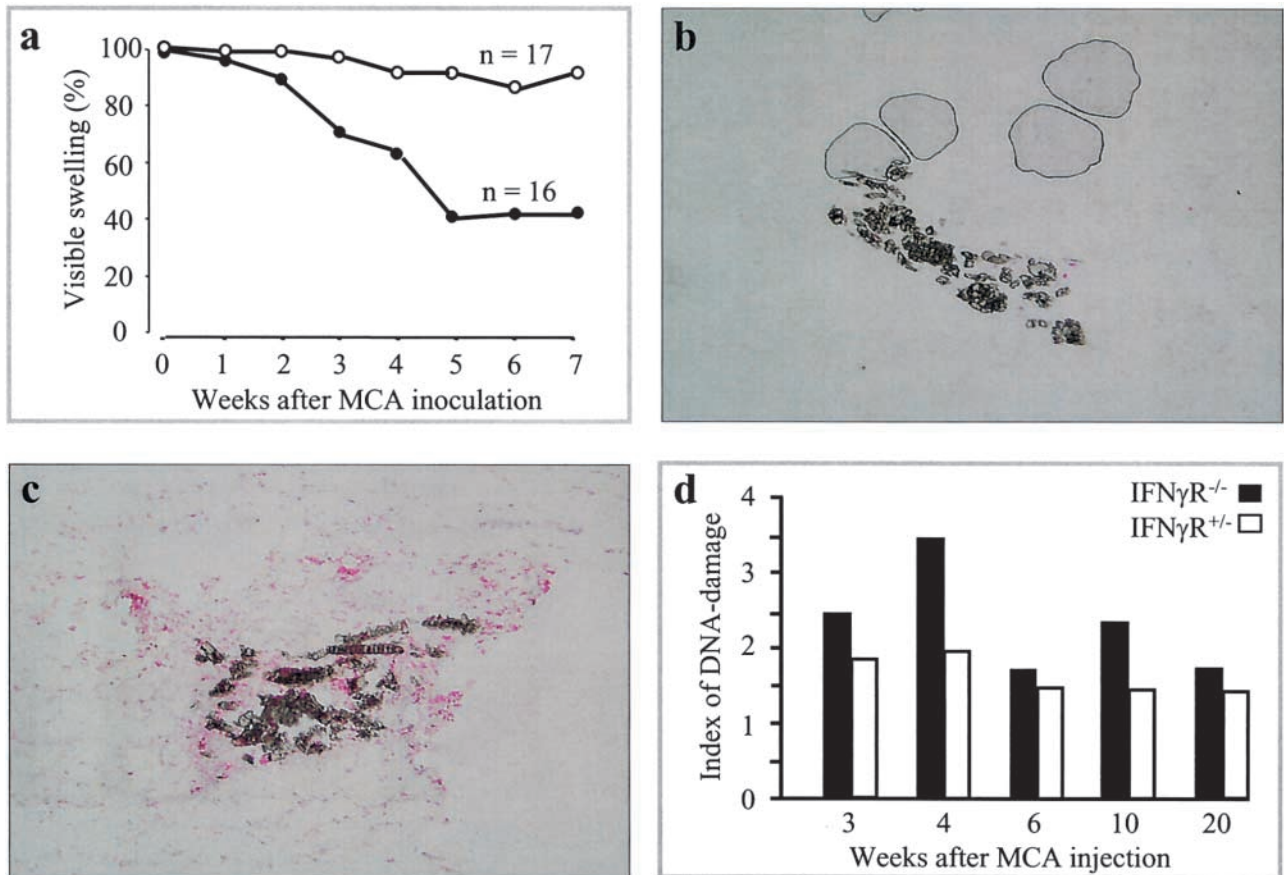


Figure 6. $\text{IFN-}\gamma\text{R}$ -dependent inhibition of MCA-diffusion and protection from tissue damage. (a) Inhibition of MCA diffusion. $\text{IFN-}\gamma\text{R}^{+/-}$ (○) and $\text{IFN-}\gamma\text{R}^{-/-}$ mice (●) were subcutaneously injected with 0.8 mg MCA in 0.2 ml sesame oil emulsion and the swelling of the injection site was monitored every week. The swelling with a size of >2 mm in diameter was recorded as positive. Shown is the percentage of mice with MCA/oil swelling visible at the injection site. Numbers of mice in each group are indicated. (b–d) Reduced tissue damage in $\text{IFN-}\gamma\text{R}^{+/-}$ compared with $\text{IFN-}\gamma\text{R}^{-/-}$ mice. Groups of mice (3–5 per time point and mouse strain; see Fig. 4 c) were injected i.m. with 0.8 mg MCA in sesame oil and tissue sections were prepared at indicated time points after injection. Tissue sections were confirmed to contain MCA (visible as black crystals) and then stained for DNA breaks by the TUNEL technique. Staining results were arbitrarily evaluated as an index for DNA-damage from 1 to 4. As an example stainings of a section from an (b) $\text{IFN-}\gamma\text{R}^{+/-}$ and (c) $\text{IFN-}\gamma\text{R}^{-/-}$ mouse 10 wk after MCA injection with an index of 1 and 4, respectively, are shown. (d) The mean indexes for DNA-damage of $\text{IFN-}\gamma\text{R}^{+/-}$ (white bar) or $\text{IFN-}\gamma\text{R}^{-/-}$ mice (black bar) at different time points after MCA injection.

pating that at a given dose some mice would not develop a tumor (Fig. 7 a). For example, if 0.1 mg MCA were injected, 82% of the mice had developed a tumor by week 24 and the remaining mice stayed tumor-free for ~ 1 y. This indicated that the process of malignant transformation was terminated. At that time, serial tissue sections of injected muscles were analyzed for the presence of MCA. In eight out of nine mice, injected 65–75 wk ago, MCA was detected (Fig. 7 b). MCA was usually embedded within a cluster of cells with an altered morphology (compare Fig. 7 b and Fig. 4 i). A collagenous capsule around MCA was observed by staining with the antifibroblast/extracellular matrix mAb ER-TR7 (Fig. 7 c) and a pan-anti-collagen mAb (Fig. 7 d). Only few inflammatory cells (Mac1⁺ and Gr1⁺) could be detected at these late time points (data not shown). Thus, MCA can persist within microscopic scars in tumor-free mice for a long time.

Discussion

Our data suggest that in the course of a tissue repair response MCA is encapsulated. This limits tissue injury as well as malignant transformation. Encapsulated MCA persists virtually life-long in tumor-free mice. The IFN- γ R^{+/-} mice control chemically induced but not spontaneous tu-

mor development. This appears to be related to IFN- γ -regulated extracellular matrix deposition. The results show a protective role of an innate inflammatory response in the early phase of chemical carcinogenesis. The foreign body reaction as a protective mechanism to control chemical carcinogenesis sheds new light on scar cancer and is distinct from Burnet's concept of immunosurveillance.

Inflammation and Cancer. There is strong evidence that inflammation can cause or contribute to cancer (8, 14). In some cancer-prone mice (e.g., transgenic for ras) induction of inflammation-induced tumors (37). In others (e.g., transgenic for polyoma middle T antigen, HPV16, or defective for the tumor suppressor gene APC), inhibition of inflammation or defects of inflammatory cells reduced tumor formation (14–16). A tumor-promoting role of inflammation during chemical skin carcinogenesis has recently been suggested, since TNF^{-/-} mice were resistant to carcinogenesis (13). These models suggest that inflammation supported tumor growth at a comparably late phase, e.g., promotion or progression. Inflammation at the site of MCA occurred during an early stage and was associated with inhibition of tumor development. The type of inflammatory cells (macrophages, granulocytes, and fibroblasts) and tissue damage at the site of MCA suggest a tissue repair response which correlated with decreased MCA diffusion,

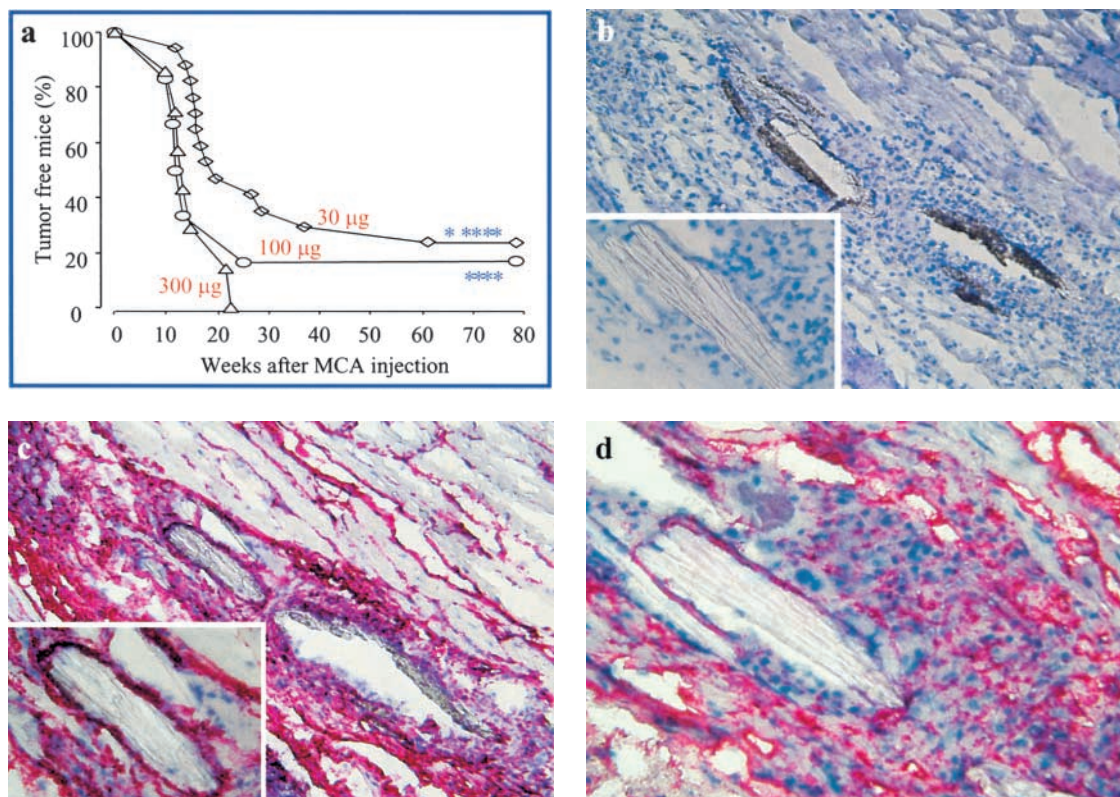


Figure 7. Encapsulated MCA persists in long-term tumor free mice. (a) BALB/c mice were injected intramuscularly with 300 μ g (Δ), 100 μ g (\circ), or 30 μ g (\diamond) of MCA in sesame oil as indicated. Tumor development was monitored every 2–3 d and mice with a tumor of ≥ 10 mm were recorded as tumor positive. Each group contained 12–16 mice. *Long-term tumor-free mice were killed at the indicated time points and the analysis of tissue sections revealed microscopic fibrosis and encapsulated MCA. (b–d) Example stainings of the encapsulated MCA 65–75 wk after injection: (b) with Hematoxylin only, (c) mAb ER-TR7 for fibroblasts and extracellular matrix and, (d) a pan-collagen specific mAb. Inlet figures in b and c show a piece of encapsulated MCA at higher magnification. Original magnifications: (b and c) $\times 100$; (d): $\times 200$.

tissue damage and malignant transformation in an IFN- γ -dependent fashion. Inflammation at the site of MCA was observed in both, IFN- γ R^{+/-} and IFN- γ R^{-/-} mice, indicating qualitative rather than quantitative differences. T cells were, at all time points, only a minority at the site of MCA, compatible with a tissue repair response. Most prominent were fibroblasts which closely surrounded MCA and might be the cell type mostly exposed to mutagenic hits. Therefore, it is not surprising that MCA typically induces fibrosarcomas. The relationship between inflammation and cancer may be more complicated than currently believed, if the stimulus for inflammation, e.g., carcinogen-induced tissue damage versus hyperplastic tissue, or the time point of inflammation are considered.

The Foreign Body Reaction. The foreign body reaction is characterized by encapsulation of foreign material. It is phylogenetically one of the oldest defense mechanisms predating adaptive immunity, a major protective mechanism in invertebrates and usually observed as a pathological reaction in humans. We found that in the course of a tissue repair response MCA was encapsulated and remained quasi life-long in tumor-free mice within microscopic scars. The high susceptibility to chemical carcinogenesis in IFN- γ R^{-/-} mice was associated with increased diffusion of MCA from the injection site and more pronounced DNA damage as shown by the TUNEL staining. Since locally produced IFN- γ -induced collagenous capsule formation, the results suggest that fibroblasts in the vicinity of MCA deposited-extracellular matrix that protected the local tissue and themselves from mutations. This process was more efficient in IFN- γ R-competent mice. It is interesting to note that MCA-induced fibrosarcomas down-regulate expression of extracellular matrix proteins (36, 38, 39). PAHs are ubiquitous environmental pollutants, they are very stable (40) and can accumulate in the lung (41-44). Since human exposure to benzo(a)pyrene has been estimated at 17 ng average daily intake from the air (45), one function of the foreign body reaction could be to protect from (frequent) tissue damage and concomitantly from (rare) malignant transformation by carcinogens.

Our results shed new light on the mechanism how tumor promoters which are not carcinogenic could enhance tumor development at PAH-treated sites after a long time period. Tumor-promoters like TPA induce inflammation. It is thought that initiated cells acquire additional mutations, because they are forced into DNA replication or through oxidative damage increased in inflamed tissues (8). A not exclusive alternative is that during tissue remodeling, an obligatory step during inflammation, extracellular matrix is degraded and MCA is set free to induce additional mutations. TPA, for example, causes a stable reduction of dermal collagen content in mouse skin which appears to be a prerequisite of tumor development (46).

G. Friedrich (47) and R. Rössle (48) found in a series of patients (frequently smokers) that lung carcinomas grew at sites of scars. They suggested that scars predisposed to cancer be termed *Narbenkrebs* (scar cancer). Scars within the carcinomas had an immature phenotype (increased col-

lagen type 3 content) indicative for an early stage of fibrotic process, whereas scars at some distance from the neoplasm revealed a mature, late stage of the fibrotic process (decreased type 3 and increased type 1 and 4 collagen) (49). Our results indicate that scars can be protective as the end product of MCA-encapsulation. Whether scar cancer results from inefficient encapsulation of carcinogen is not yet known, however, benzo(a)pyrene was detected in substantial amounts in lung tissues of smokers (41, 44) and former smokers retain a substantial risk of developing lung cancer (50).

The Role of IFN- γ /IFN- γ R during Protection from Chemical Carcinogenesis. Two results at least partially explain the role of IFN- γ /IFN- γ R during inhibition of chemical carcinogenesis. First, MCA diffused more rapidly from the injection site in IFN- γ R^{-/-} compared with IFN- γ R^{+/-} mice which is reminiscent to the phenotype of IFN- γ R deficiency in humans. These patients have a selective defect in granuloma formation. Disseminated diseases occurred when they were infected with mycobacteria and perhaps also other intracellular bacteria (51, 52). Granuloma formation is an early event during mycobacteria infection which involves encapsulation promoted by fibrosis (53). Since the patients apparently respond normally to other infections which do not involve granuloma formation, e.g., against viruses, it is possible that the defective granuloma formation due to IFN- γ R deficiency led to ineffective encapsulation and bacterial dissemination. Second, the local IFN- γ production led to extracellular matrix accumulation and encapsulation of IFN- γ -producing cells. Whether this is a direct or indirect effect of IFN- γ is not known. For inhibition of chemical carcinogenesis IFN- γ R expression by either hematopoietic or nonhematopoietic cells was sufficient. This indicates that cells from both fractions can be induced by IFN- γ to produce factors involved in extracellular matrix accumulation. For example, IFN- γ upregulates PDGF and PDGF-receptor expression by vascular smooth muscle cells during arteriosclerosis (31). Similarly, IFN- γ induces PDGF expression by monocytes/macrophages.

A Mechanism Different from Immunosurveillance. Burnet proposed that cells derived from a 'thymus-dependent system' recognize and eliminate frequently arising 'potentially dangerous mutant cells', termed immunosurveillance (54). In modern terms it postulates that T cells spontaneously recognize an immunogenic, e.g., mutant, peptide in a MHC-restricted fashion, become activated and destroy the tumor cells. Immunosurveillance against virus-associated tumors has been demonstrated (55), but it does not seem to play a role in preventing the development of most forms of cancers induced by chemical or physical carcinogens (56). Our results support the existence of a spontaneous mechanism controlling MCA-induced tumor development that, however, differs from immunosurveillance. It is operative during MCA-induced carcinogenesis but not during spontaneous tumor development (by p53 deficiency). Even though we cannot exclude the participation of T cells, we have no indications for their involvement. We could detect only few T cells at the site of MCA during the first 20 wk

and we did not see signs of immuneselection in IFN- γ R-competent mice. Importantly, nude mice lacking thymus-dependent T cells and SCID mice lacking B cells and all T cells (α/β^+ , γ/δ^+ , and NK T cells) do not develop MCA-induced tumors faster or more frequently (57, 58). A coincidental finding in the latter study was that, despite similar growth kinetics, tumors from SCID mice grew less frequent upon transplantation into wild-type mice. This argues against the concept of immuneselection but indicates that the tumor is conditioned in the primary host for subsequent growth in recipient mice. Prehn and Bartlett had already shown that there existed no strict correlation between latency period of MCA-induced tumors and immunogenicity tested by tumor transplantation experiments (59). Our preliminary data of MCA-induced carcinogenesis in CB17-SCID and recombination activation gene 1-deficient mice showed a 3–4 wk delay in tumor development compared with control CB17 and C57Bl/6 mice (data not shown). Since we did not use control litter mates in these experiments, a contribution of T cells has to be investigated. Indeed, a little difference in tumorigenesis (3–4 wk) could also be found when the same inbred mice from different sources were compared (data not shown). It is known that the genetic background can dramatically change susceptibility to chemical carcinogenesis independent of immunocompetence (2, 60).

Tumor transplantation immunity is usually mediated by T cells. It is not clear whether transplantable tumor models can explain the mechanism by which MCA-induced carcinogenesis is inhibited. Since bone marrow chimera experiments argue against this possibility (Fig. 2 and reference 30), we analyzed the local events in the MCA-treated host. This revealed a tissue repair response that can occur in the absence of T cells. Therefore, our data suggest that MCA-induced carcinogenesis is controlled by a mechanism different from immunosurveillance and that the target for tumor inhibition is primarily MCA and not tumor cells. Prevention of tumor development by encapsulation of mutagens should be more effective than killing transformed cells.

While this study was in progress, other studies showed that tumor development induced by MCA was increased in perforin-, IFN- γ , IFN- γ R-, TCR- α 281 (NK T cell), and recombination activation gene 2-deficient mice during an observation period of up to 33 wk (61–65). Based on tumor transplantation experiments, the authors suggested a mechanism related to immunosurveillance. We do not want to exclude this possibility, however, it needs to be shown in the primary MCA-treated host that a tumor appeared and was subsequently eliminated. Kaplan et al. (62) found an increased incidence of tumors in p53^{-/-} mice if IFN- γ R was also deficient. Since we did not find any difference between p53^{-/-}/IFN- γ R^{+/-} compared with p53^{-/-}/IFN- γ R^{-/-} mice, we also compared tumor incidence between p53^{+/-}/IFN- γ R^{+/-} and p53^{+/-}/IFN- γ R^{-/-} mice. These mice develop tumors after a long latency period and should reveal a change in tumor incidence or kinetics more easily, but again no difference was observed. We could also not detect significant differences

in the tumor spectrum between these groups. Although the reason for the discrepancy is not clear, the mixed genetic background of p53-deficient mice (C57Bl/6 \times 129Sv/Ev) could be a problem. It has been shown that 129Sv and C57Bl/6 \times 129Sv p53^{-/-} mice differ in tumor kinetics and spectrum (32). In conclusion, the relative contribution of immunosurveillance versus the mechanism proposed here requires further investigations, e.g., to answer the question why recombination activation gene 2^{-/-} but not SCID mice, two strain with similar phenotype, have increased susceptibility to MCA-induced carcinogenesis (58, 64). Our results suggest that the primary mechanism which controls chemical carcinogenesis is a tissue repair response and foreign body reaction during which IFN- γ R plays a not yet completely understood role.

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