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19. Stationary D4 cells (wet weight, 300 g) were resuspended in breaking buffer [50 mM tris-HCl (pH 7.5), 0.008% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT); leupeptin (1 μg/ml), pepstatin (1 μg/ml), aprotinin (1 μg/ml), phenylmethylsulfonylfluoride (PMSF) (0.125 μg/ml), 1 mM NaN_3; 300 ml]. The next steps were done between 0° and 4°C. The cells were broken with 0.5-mm glass beads in a bead-beater (BioSpec Products). The cell lysate was clarified by centrifugation (40,000*g*, 45 min). Polyethyleneimine (PEI) was added (0.3% final concentration), and the mixture was stirred on ice for 15 min. The precipitate was removed by centrifugation (40,000*g*, 15 min). Ammonium sulfate was added to the supernatant (45% final concentration), and the mixture was stirred on ice for 1 hour. The precipitate was separated from the supernatant by centrifugation (40,000*g*, 45 min) and resuspended in 1.5 liters of PC buffer [breaking buffer with 50 mM sodium glutamate (pH 9.5) replacing the tris-HCl]. A thick slurry of phosphocellulose (80 ml) was added, and the mixture was incubated with gentle agitation for 1 hour. The phosphocellulose was separated from the supernatant by low-speed centrifugation and packed into a glass column. The column was washed and eluted with PC buffer at pH 12. The pH of the eluted protein was reduced to pH 5.5 with 10% acetic acid. The sample was clarified by centrifugation (40,000*g*, 30 min) and loaded onto a MonoQ column (Pharmacia) equilibrated in PC buffer at pH 5. The p40 protein was eluted with a linear gradient to 100 mM NaCl. Active fractions were pooled and loaded onto a ProRPC column (Pharmacia). The p40 protein was eluted with a 20 to 35% acetonitrile gradient in 0.1% trifluoroacetic acid.
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21. H17C1A1 (gift of L. Hartwell) cells arrested in S phase were suspended in an equal volume of 80 mM β-glycerol phosphate, 20 mM Hepes (pH 7.5), 50 mM NaF, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 1 mM DTT, 300 μM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml) and broken by vortexing with 0.5-mm glass beads. The lysate was clarified by centrifugation (40,000*g*, 45 min). PEI was added to the supernatant (0.3% final concentration) and the mixture was incubated on ice with gentle stirring for 15 min. The precipitate that formed was removed by centrifugation (40,000*g*, 15 min), and the supernatant was applied to a Q-Sepharose column equilibrated in Q buffer [20 mM β-glycerol phosphate, 20 mM Hepes (pH 7.5), 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml)]. Active p34 was eluted with a linear salt gradient to 500 mM NaCl. Ammonium sulfate (45% final concentration) was added to the pooled active fractions, and the mixture was stirred on ice for 1 hour. The precipitate was removed by centrifugation (40,000*g*, 45 min), resuspended in a minimal volume of Q buffer, and further purified by gel filtration on Superose 12.
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The Prevention of Thymic Lymphomas in Transgenic Mice by Human O^6-Alkylguanine-DNA Alkyltransferase

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Nitrosoureas form O^6-alkylguanine-DNA adducts that are converted to G to A transitions, the mutation found in the activated *ras* oncogenes of nitrosourea-induced mouse lymphomas and rat mammary tumors. These adducts are removed by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase. Transgenic mice that express the human homolog of this protein in the thymus were found to be protected from developing thymic lymphomas after exposure to *N*-methyl-*N*-nitrosourea. Thus, transgenic expression of a single human DNA repair gene is sufficient to block chemical carcinogenesis. The transduction of DNA repair genes in vivo may unravel mechanisms of carcinogenesis and provide therapeutic protection from known carcinogens.

DNA damage is an initiating event in chemical carcinogenesis that leads to mutations in proto-oncogenes and tumor suppressor genes (*1*). Subsequent induction of error-prone DNA repair and cell proliferation may increase the rate of secondary mutations, thereby accelerating neoplastic transformation (*2*). These early events in chemical carcinogenesis have been extensively studied in animal tumors induced by *N*-methyl-*N*-nitrosourea (MNU) (*3*). Like other *N*-nitroso compounds, MNU methylates DNA at a number of sites, including the O^6 position of guanine (*4*). The resulting O^6-methylguanine-DNA adduct is converted to a G → A point mutation during DNA synthesis (*5*). MNU also induces other events that may be carcinogenic, including DNA strand breaks, sister chromatid exchanges (6), and cell proliferation within the target tissue (*7*, *8*). Although any of these forms of genotoxic damage could act as the initiating event, persistent O^6-methylguanine adducts have been proposed to be the critical lesion in a variety of MNU-induced experimental tumors, such as mouse lymphomas and rat mammary tumors, because these tumors frequently display G → A activating mutations in K-*ras* or H-*ras* (9).

O^6-alkylguanine-DNA adducts are removed by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (E.C. 2.1.1.63; alkyltransferase) (*10*), which transfers the alkyl group from the O^6 position of guanine to a cysteine residue in its own active site and becomes irreversibly inactivated in the process (*11*). The capacity for O^6-methylguanine adduct repair by this mechanism is therefore directly proportional to the number of alkyltransferase molecules in the cell. A correlation exists between alkyltransferase activity and nitrosourea-induced cytotoxicity and mutagenicity in vitro (*6*, *12*). Manipulation of alkyltransferase concentrations alters cellular resistance to nitrosoureas. Thus, specific alkyltransferase inhibitors increase the cytotoxicity and the frequency of mutations and sister chromatid exchanges induced by nitrosoureas (*13*), whereas transduction of the alkyltransferase gene *MGMT* into cells without endogenous alkyltransferase activity reduces cytotoxicity and the frequency of G → A mutations (*13*, *14*). Tissues susceptible to the carcinogenic effect of nitrosoureas, such as thymus, breast, bone marrow, kidney, and brain, have 3 to 18 times less alkyltransferase activity than tissues that are not susceptible to these agents (*3*, *15*).

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In a number of genetic diseases, defects in DNA repair have been linked to an increased risk of cancer (*16*), which implies that correction of these defects may reduce cancer risk. To test this hypothesis, we investigated whether enhanced alkyltransferase-mediated repair of O^6-methylguanine adducts in transgenic mice would block tumor initiation by nitrosoureas. Thymic lymphomas are the most prevalent MNU-induced tumors in mice and occur in 20 to 80% of mice with a latency of 70 to >200 days (*3*, *9*). These lymphomas originate from T cells and invade the thymus and spleen, resulting in massive organomegaly (*15*). Mouse thymus has low endogenous alkyltransferase activity and a high frequency of persistent O^6-methylguanine adducts after MNU exposure (*3*), which suggests that deficient DNA adduct repair is responsible for the initiation of carcinogenesis.

To study the effect of increased alkyltransferase activity on nitrosourea-induced tumors, we generated transgenic mice that expressed high concentrations of the human alkyltransferase gene *MGMT* in the thymus ($MGMT^+$). The strain used was the F_1 cross between C57BL/6 and SJL mice (*8*, *17*). To target *MGMT* expression to the thymus, we constructed a chimeric gene that consisted of the avian β-actin promoter region, the *MGMT* cDNA cloned from the VACO 6 human colon carcinoma cell line (*18*), the polyadenylate [poly(A)] region from the bovine growth hormone (bGH) gene (*19*), and the human CD2 locus control region (*20*) (Fig. 1).

Twenty-three mice were screened for the transgene. Southern (DNA) analysis of genomic DNA digested with Hind III or Sac I and hybridized with a ^{32}P-labeled *MGMT* cDNA probe revealed that three mice carried the intact gene. Two of them transmitted the gene in a heterozygous fashion, which indicates germ line insertion. One mouse, Founder 11 (Fo11), carried five copies of the gene in a head-to-tail concatemer and consistently transmitted expression of the transgene to offspring. The other heterozygote carried over 100 gene copies, but none of its offspring expressed the gene. The third mouse was mosaic but also transmitted *MGMT* expression to offspring.

Offspring of Fo11 were produced by backcrossing the male founder with (C57BL/6 × SJL)F_1 female mice. *MGMT* expression was analyzed by Northern (RNA) blot analysis of total cellular RNA probed with human *MGMT* cDNA (Fig. 2A). The transgene was abundantly expressed in thymus tissue, which indicates that the CD2 locus control region appropriately targeted transgene expression to T cells. *MGMT* mRNA was also expressed in muscle (because of the β-actin promoter) and in spleen. No *MGMT* mRNA was detected in liver, and trace amounts were detected in kidney.

Total cellular alkyltransferase activity was measured in tissues from both $MGMT^+$ transgenic mice and their nontransgenic littermates. Alkyltransferase activity was markedly increased in the thymuses, spleens, and muscles of the transgenic mice, reaching values that were 11 to 88 times higher than those seen in the nontransgenic mice (Table 1). The greatest increase in activity occurred in thymus tissue (47 fmol of activity per microgram of DNA in transgenic mice compared to 0.62 fmol of activity per microgram of DNA in nontransgenic mice) (Table 1). In transgenic mice, alkyltransferase activity in the thymus was almost eight times higher than that in the liver, the tissue with the highest endogenous alkyltransferase activity (*15*). Alkyltransferase activity in the livers and kidneys of the $MGMT^+$ transgenic mice was slightly elevated compared to the nontransgenic values, which indicates that *MGMT* was expressed in small amounts in these tissues. SDS–polyacrylamide gel electrophoresis (PAGE) of cell extracts incubated with [^3H]methylated DNA to label the alkyltransferase showed that the labeled protein corresponded in size to the human homolog [relative molecular mass (M_r) = 22,000] (Fig. 2B) and was slightly smaller than the endogenous murine alkyltransferase protein (M_r = 24,000) (*21*). The *MGMT* transgene was consistently expressed in the thymuses and spleens from all $MGMT^+$ transgenic offspring of Fo11.

To determine whether thymic expression of human *MGMT* would prevent MNU-induced thymic lymphomas, we treated $MGMT^+$ transgenic mice and their nontransgenic littermates at 6 weeks of age with MNU (either 50 or 80 mg per kilogram of body weight) by intraperitoneal injection (Fig. 3). Mice that developed thymic lymphomas typically had respiratory distress and at autopsy showed massive thymic tumors encasing the heart and lungs, splenomegaly, a lymphoblastic leukemia, and invasion of tumor cells into many other

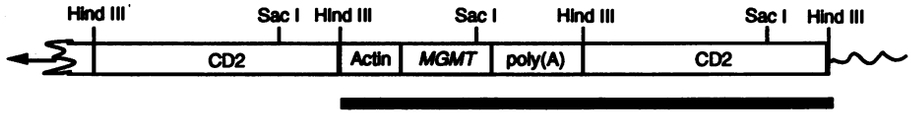

Fig. 1. MGMT transgenic construct. The *MGMT* gene was cloned from the VACO 6 human colon cancer cell line (*18*) by reverse transcriptase–polymerase chain reaction (*29*). A 2.0-kb fragment of the CD2 locus control region was used to target expression to the thymus (*20*). The *MGMT* transgene is shown as it would appear in a tandem array in the genome. The shaded bar indicates a single copy of the chimeric gene, and the CD2 region to the left indicates part of a second copy of the gene. Actin, the β-actin promoter; *MGMT*, the human *MGMT* cDNA; poly(A), the bGH exon 5 and poly(A) region; CD2, the CD2 locus control region. The sites recognized by restriction enzymes Hind III and Sac I used to determine the orientation and integrity of the insertion are indicated.

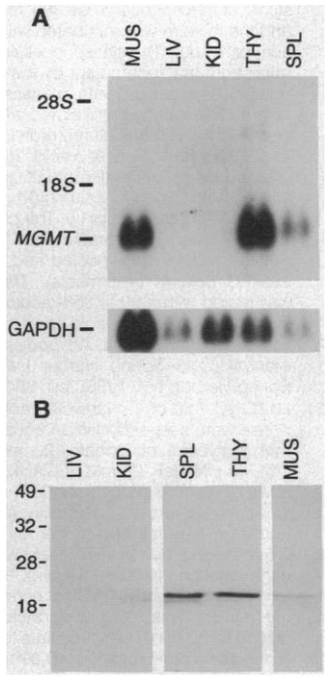

Fig. 2. Expression of *MGMT* RNA and protein in transgenic mice. (**A**) Total cellular RNA was prepared (*19*) from various tissues of Fo11 F_1 $MGMT^+$ offspring, separated by formaldehyde-agarose gel electrophoresis (10 μg per lane) (*19*), and subjected to Northern blot analysis with a ^{32}P-labeled *MGMT* cDNA probe. The expected size of the β-actin–MGMT transcript is 1.4 kb. The blot was washed and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to evaluate differences in loading between lanes. LIV, liver; THY, thymus; KID, kidney; SPL, spleen; MUS, muscle. Human *MGMT* cDNA does not cross hybridize with endogenous mouse *MGMT* mRNA. (**B**) We incubated tissue extracts with [^3H]methyl-DNA as in the alkyltransferase assay (*11*, *15*, *19*) to label the alkyltransferase through the methyl transfer reaction, and the proteins were separated by SDS-PAGE (*19*). The human alkyltransferase (M_r = 22,000) is the dominant protein band seen. Although the bands reflect the transfer of [^3H]methyl groups to the alkyltransferase, band intensity is not linear because in samples with high enzyme activity [thymus and spleen; abbreviations are as in (A)], the substrate [^3H]-methyl-DNA has been exhausted. A faint band corresponding to the endogenous murine alkyltransferase (M_r = 24,000) was seen in liver. Numbers at the left indicate the M_r (× 10^{-3}) of protein standards (Bio-Rad).

tissues, including liver, brain, kidney, and lymph nodes. The tumor cells were intermediate and large noncleaved cells, consistent with a lymphoblastic lymphoma. A smaller number of mice developed a less aggressive splenic lymphoma similar to the lymphoma that develops spontaneously in SJL mice (*8, 17*). Lymphomas developed between 101 and 363 days (median = 139 days) at the 50 mg/kg dose and between 75 and 227 days (median = 109 days) at the 80 mg/kg dose.

A total of 33 lymphomas were observed: 24 were thymic with involvement of multiple tissues and 9 involved only the spleen (Fig. 3). Twenty-three of 24 thymic lymphomas occurred in nontransgenic mice (58% versus 4%, $P < 0.0001$ by chi-square analysis), whereas six nontransgenic and three MGMT$^+$ transgenic mice developed lymphomas only in the spleen. The single thymic lymphoma seen in the MGMT$^+$ transgenic group occurred in a mouse given MNU at a dose of 80 mg/kg and was detected 145 days after treatment and 13 days after the last lymphoma occurred in the nontransgenic group (Fig. 3C). *MGMT* expression in this lymphoma was about 10% of that expected, which raises the possibility that down-regulation had increased the sensitivity of this mouse to MNU. In mice that received MNU at a dose of 80 mg/kg, two MGMT$^+$ transgenic mice developed lymphomas only in the spleen (Fig. 3D), whereas none were observed in nontransgenic mice. In the 50 mg/kg group (Fig. 3, A and B), only one MGMT$^+$ transgenic mouse developed a lymphoma and it was splenic in origin and occurred late (at 267 days). Overall, lymphomas developed in 16% (4 of 25) of the MGMT$^+$ transgenic mice and in 71% (29 of 41) of their nontransgenic MNU-treated littermates [$P < 0.00005$].

These results provide strong evidence that DNA repair in general, and O^6-methylguanine-DNA adduct repair in particular, is the first line of defense against certain types of chemical carcinogens. The possibility that resistance to tumor induction was an artifact of the insertion site of the transgene, perhaps disrupting a susceptibility locus, is unlikely for two reasons. (i) Offspring of a third MGMT$^+$ founder that expresses *MGMT* in the thymus were also protected from MNU-induced lymphoma. (ii) Radiation-induced lymphomas occurred in both MGMT$^+$ and nontransgenic littermates at equal frequency (*22*). Consequently, the absence of alkyltransferase-mediated repair of O^6-methylguanine-DNA adducts, as opposed to other forms of DNA damage and repair, appears to be the critical determinant of MNU-induced carcinogenesis in the thymus, as predicted by Pegg (*23*).

It is striking that increasing the activity of a single DNA repair process reduced the overall incidence of MNU-induced lymphomas from 71 to 16%, reduced the incidence of thymic lymphomas from 58 to 4%, and increased the latency of the lymphomas that did occur. The single thymic lymphoma in an MGMT$^+$ transgenic mouse could represent either a breakthrough in protection as a result of heterogeneous expression of the MGMT transgene or an alternative pathway of carcinogenesis induced by less common mutagenic DNA adducts, such as O^4-methylthymine (*4*). The similar incidence of splenic lymphomas in the two groups of mice may reflect the inability of the *MGMT* transgene to protect against carcinogenesis in the splenic B lymphocyte [the cell of origin of these lymphomas (*8, 17*)]. Susceptibility to splenic lymphomas may have been uncovered in the MGMT$^+$ transgenic mice because they did not die from thymic lymphomas.

These results suggest that the rate-limiting step in cancer initiation for other carcinogens, including procarbazine, nitrosamines, dacarbazine, and the chloroethylnitrosoureas (*10, 15, 23, 24*), may be formation of persistent, unrepaired O^6-alkylguanine adducts. Other experimental tumors that may be prevented by the overexpression of *MGMT* include nitrosamine-induced lung and liver tumors, MNU-induced mammary tumors, and ethylnitrosourea-induced brain tumors (*3, 9, 25*).

Tissue-specific alkyltransferase deficiency increases susceptibility to nitrosourea-induced toxicity and malignancy. In clinical trials, the toxicity of nitrosoureas to human hematopoietic cells is dose-limiting (*26*). These cells contain smaller amounts

Table 1. Alkyltransferase activity in transgenic mice. Tissues were prepared from four to seven mice, 6 to 8 weeks old, from each group and assayed for alkyltransferase activity (*15, 19*). One femtomole of activity was defined as the removal of 1 fmol of O^6-methylguanine from methylated DNA (*15, 19*). Tissue samples from each mouse were assayed two to three times. Data are presented as the mean ± SE for each group of mice.

Tissue	Alkyltransferase activity (fmol/mg protein)		Alkyltransferase activity (fmol/μg DNA)	
	Nontransgenic	MGMT$^+$ transgenic	Nontransgenic	MGMT$^+$ transgenic
Thymus	50 ± 13	4393 ± 641	0.62 ± 0.15	47 ± 7.6
Spleen	42 ± 9.4	1280 ± 340	0.63 ± 0.18	22 ± 5.3
Muscle	7 ± 4	128 ± 26	0.90 ± 0.6	10 ± 2.3
Liver	80 ± 27	150 ± 18	6.2 ± 1.3	8.9 ± 1.7
Kidney	25 ± 13	116 ± 53	1.4 ± 0.5	4.4 ± 1.8

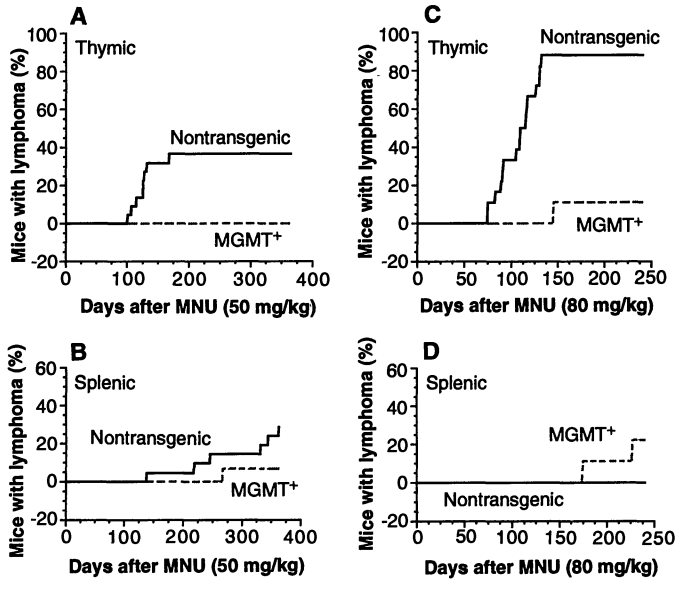

Fig. 3. Prevention of MNU-induced lymphomas in MGMT$^+$ transgenic mice. Incidence of thymic lymphomas (**A** and **C**) and lymphomas only in the spleen (**B** and **D**) observed in nontransgenic (solid line) and MGMT$^+$ transgenic (dashed line) mice. Littermate offspring of a cross between Fo11 and (C57BL/6 × SJL)F_1 mice were treated with MNU intraperitoneally at 6 weeks of age. MGMT$^+$ transgenic mice were identified by slot-blot analysis of tail DNA obtained at 2 weeks of age, but the results were not known at the time of MNU injection. A total of 23 nontransgenic and 15 MGMT$^+$ transgenic mice were treated with MNU (50 mg/kg), and a total of 18 nontransgenic and 10 transgenic mice were also treated (80 mg/kg). Mice were killed and autopsied when symptoms of lymphoma or other malignancy developed. In nontransgenic mice, the thymic lymphomas invaded other tissues, including spleen (15 cases), lymph nodes (13), liver (12), kidney (10), and brain (3). Eight mice had lymphoblastic leukemia. One MGMT$^+$ transgenic mouse developed a soft tissue sarcoma and one nontransgenic mouse developed an osteogenic sarcoma.

of alkyltransferase than T cells or other human tissues and, after chemotherapy, rapidly proliferate to renew the progenitor compartment (*7*, *8*, *11*, *15*), thereby increasing the risk of mutagenesis. Thus, inadequate amounts of alkyltransferase may be responsible for the development of secondary acute myeloid leukemias in patients who receive chloroethylnitrosourea- or procarbazine-based chemotherapy (*27*). In view of our findings with experimental tumors, a clinical strategy in which MGMT is introduced into hematopoietic precursors by gene therapy methods may merit consideration. Efficient expression of MGMT may increase bone marrow resistance to the cytotoxic and leukemogenic effects of nitrosoureas and potentially benefit patients receiving these agents.

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20. The chimeric gene was constructed as follows. (i) The 340-bp avian β-actin promoter was isolated from pUC18 β-actin (*28*) and inserted into the unique Sal I site of plasmid Cla12N (*28*). (ii) The 702-bp *MGMT* cDNA was inserted into the Bam HI site of Cla12N. (iii) The 710-bp Sma I–Eco RI fragment from the bGH gene, which includes a portion of the fifth exon and the poly(A) region (*19*), was inserted into the Sma I–Eco RI sites of Cla12N. (iv) The 1903-bp Cla I fragment with all of these sequences was ligated into a Bluescript M13 plasmid containing a 2-kb Hind III fragment of the human CD2 locus control region [D. R. Greaves, F. D. Wilson, G. Lang, D. Kioussis, *Cell* **56**, 979 (1989)]. The 3933-bp fragment containing the transgene was isolated after digestion with Apa I and Spe I. Purified DNA (50 ng/ml) was microinjected into single-cell embryos from pregnant (C57BL/6 × SJL)F_1 mice 8 hours after fertilization. The embryos were then reimplanted into pseudopregnant mice. We predicted that the β-actin promoter would allow expression of the transgene in a number of tissues, that the bGH poly(A) region would confer mRNA stability (*19*), and that the CD2 locus control region would target expression to T cells in the thymus, as shown for the β-globin–CD2 chimeric gene.
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Terrestrial Soft-Bodied Protists and Other Microorganisms in Triassic Amber

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Protozoa, cyanobacteria, sheathed algae, sheathed fungi, germinating pollen or spores, and fungal spores have been found in amber 220 to 230 million years old. Many of these microorganisms can be assigned to present-day groups. This discovery of terrestrial, soft-bodied protists that can be referred to modern groups indicates that morphological evolution is very gradual in many protists and that both structural and probably functional stasis extend back at least to the Upper Triassic period.

The majority of organisms found in amber have been arthropods that accidently fell into the sticky sap of resin-bearing trees (*1–3*). During an examination of Triassic amber from southern Germany, we discovered well-preserved microorganisms, which formed a biocenose in association with the resin-bearing plant. These microorganisms represent the earliest known soft-bodied, terrestrial protists. Small pieces of amber were removed from layers of Raibler Sandstone on Mount Leitnernose in Schliersee, Bavaria, Germany. These deposits belong to the Carnian stage of the upper Keuper succession of the Upper Triassic period and are dated at 220 to 230 million years old (*4*).

For microscopic observations, small pieces of the amber were crushed, mounted

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