spectrum showing aromatic, hydroxyl (or amino), and carbonyl groups. This fraction, on alumina, was resolved further; a subfraction was eluted with a mixture of petroleum ether and benzene (1 : 1) which, on gas chromatographic analysis (2.4 m by 6.4 mm column of 20 percent SE-30 on Chromosorb W, 180°C for 15 minutes, and programmed at 8°C per minute to 265°C), showed three peaks emerging at 13, 26, and 34 minutes, respectively.

The infrared spectrum of the component eluting at 13 minutes was suggestive of an aromatic ether. Absorption at 9.57 μ indicated an ether group, and peaks at 3.45, 10.69, and 13.85 μ revealed a possible methylenedioxy-substituted benzene ring (*4*). Strong absorption at 6.12 μ showed *exo* unsaturation which was substantiated by bands at 10.08 and 10.92 μ (vinyl group). Aromatic absorption was apparent at 12.09 μ (one adjacent free hydrogen on the aromatic ring) and in the usual range, 3.25 to 3.40 μ. The absence of absorption at 6.29 μ indicated the lack of conjugation of the vinyl group with the aromatic ring. The ultraviolet spectrum confirmed the presence of aromaticity (broad absorption at 260 to 295 mμ). The mass spectrum showed a parent peak at 192 and major fragment peaks at 161 (loss of -OCH_3) and 165 (substituted tropylium ion). Comparison of all spectra with those of authentic myristicin (*5*) showed identical characteristics except for two extraneous peaks (mass/charge 153, 194) in the mass spectrum of the unknown; these peaks were probably derived from a minor contaminant structurally related (for example allyl 2,6-dimethoxyphenyl ether) or dissimilar (as a substituted indole) to myristicin. Indoles and carbazoles have been isolated from the fraction containing myristicin in the original column chromatography. Similar retention times were obtained with the isolated substance and authentic myristicin on 20 percent Apiezon L on Chromosorb W (2.4 m by 6.4 mm column operated at 275°C; retention time, 7 minutes). In addition, co-chromatography of the isolated substance and authentic myristicin on SE-30 gave a single peak. The level of myristicin in cigarette smoke is at least 0.64 μg per cigarette based on the observed recovery, which was undoubtedly not quantitative.

The nitromethane-soluble fraction of smoke is of special interest since it contains the major carcinogenic polynuclear aromatic hydrocarbons of smoke and has significant physiological activity. In addition to the carcinogens this fraction contains a number of other aromatic compounds, such as benzyl benzoate and benzyl cinnamate; certain heterocyclic aromatic compounds (indoles, carbazoles, etc.) have also been demonstrated (*6*). However, myristicin appears to be unlike these recently isolated compounds in that some distinct pharmacological activity has been attributed to it. Although controversy exists as to whether the physiological effects of nutmeg oil (nauseau, tachycardia, cyanosis, stupor, and others) are due exclusively to myristicin (*2*), it appears safe to conclude, on the basis of available biological data (*2*), that myristicin has some degree of toxicity and produces some neurological effects on administration. Also, it should be noted that myristicin is an analog of safrole, which is regarded as a low-grade hepatic carcinogen for rats (*7*). Whether the low level of myristicin in cigarette smoke contributes to the overall physiological effect of smoke is unknown.

Since commercial American cigarettes contain flavoring additives, including natural oils and resins, the possibility exists that myristicin, as well as the other benzyl esters in smoke, is derived from this source rather than the tobacco leaf. Myristicin has been isolated from the oil of several species, and the benzyl esters are common constituents of many natural oils and resins (*8*).

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Homograft Target Cells: Contact Destruction in vitro by Immune Macrophages

Abstract. *Specific adherence of immune macrophages to monolayers of target cells is a passive phenomenon which represents only the first step in the mutually destructive interaction of immune macrophages and target cells. A specific hemagglutinin, responsible for specific adherence, was eluted from well-washed immune macrophages by heat treatment. The nature of the events in the interaction subsequent to adherence are unknown, but apparently demand the biosynthetic activities of the immune macrophage.*

Peritoneal macrophages from C57B1/Ks mice (*1*) immunized against antigens of A/Jax mice cause specific "contact destruction" in vitro on monolayers of specific target cells derived from A/Jax mice and C_3H mice (*2*). Our results established that: (i) the first step in the interaction is specific adherence of essentially all of the immune cells to the target cell monolayer, (ii) both the macrophage and the target cell are destroyed in the interaction, and (iii) normal peritoneal macrophages treated with specific humoral isoantibody derived from immune donors do not interact with target cells to produce cell destruction (*2*).

In our studies, immune macrophages were collected, and a standard suspension containing 20×10^6 cells per milliliter was prepared in tissue culture medium (*2*). The preparations were diluted in the medium to give suspensions containing 20, 15, 10, 5, 1, 0.5, and 0.1×10^6 cells per milliliter. A drop of each cell suspension (0.05 ml) was placed at each of two separate sites on pure monolayers of A/Jax fibroblasts or L-cells. After 48 hours the monolayers were stained by the plaque-staining method (*2*). The results from two separate experiments in which four sites were used for each concentration of immune overlay cells are reported.

An overlay of 50,000 immune cells caused full plaque formation. However, macroscopically evident partial plaques were produced with 25,000 immune cells, and microscopically evident plaques were produced with as few as 5000 immune cells. Ninety-eight percent of the cells that became detached from the plaque area were nonviable.

Starch-induced macrophages from

nonimmune C57Bl/Ks mice were collected as described (*2*). A drop of a suspension containing either 3, 2.5, 2, 1.5, 1, or 0.5×10^6 nonimmune cells was placed at each of two separate sites on each of four monolayers of target cells, either A/Jax fibroblasts or L-cells. No gross evidence of plaque formation was observed after 48 hours at any of the overlay sites. However, microscopic observations revealed that a few of the target cells in the overlay areas that received 2.5×10 nonimmune cells were clumped. When the number of nonimmune overlay cells was increased to 3.0×10^6, essentially all target cells were clumped. Although gross plaques were not produced with large numbers of nonimmune cells, some killing of target cells occurred, presumably because of nonspecific effects resulting from excessive numbers of overlay cells.

The capacity of various nonspecific immune macrophages to adhere to target monolayers of A/Jax fibroblasts and L-cells was tested. Each of five C57Bl/Ks mice was injected intraperitoneally on days 1, 5, 6, and 8 with the cells from one-fourth of the spleen and one-fourth of the liver of an inbred Swiss-Webster mouse. A second group of five mice was treated in a similar manner with spleen and liver cells from BALB/C mice. Each animal of the above groups also received an intraperitoneal injection of 1.0 ml of a 3.0-percent starch solution (*3*) in saline on day 6. The peritoneal cells were collected on day 10 from animals of both groups, and a standard macrophage-rich suspension was prepared. Each of a third group of 10 C57Bl/Ks mice received an intraperitoneal injection of 0.2 mg of heat-killed bacillus of Calmette and Guerin (BCG) suspended in Bayol F (*4*). Forty days later each of the 10 animals received an intraperitoneal injection of 0.1 mg of heat-killed BCG in saline. Twenty hours later the peritoneal cells were collected, and a standard macrophage-rich suspension was prepared.

Tests with the above preparations showed that limited adherence, restricted to a few cells, took place after 20 to 30 minutes. The time required for adherence was similar to that required for adherence of nonimmune cells on target monolayers.

The capacity of various nonspecific immune cells to form plaques was tested by placing a drop of 2.5×10^6 cells at each of two sites on each

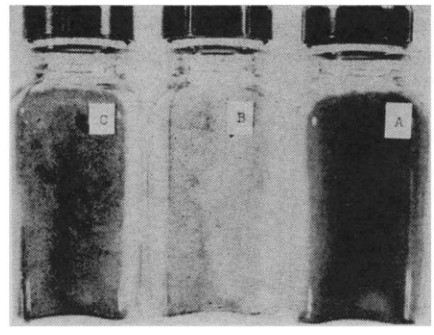

Fig. 1. (*A*) L-cell monolayer 48 hours after treatment at two sites (2.5×10^6 cells per site) with macrophages derived from C57Bl/Ks mice immunized with BALB/C cells. (*B*) A/Jax fibroblast monolayer 48 hours after treatment at two sites (1×10^6 cells per site) with macrophages derived from C57Bl/Ks mice immunized with Swiss-Webster cells. (*C*) A/Jax-fibroblast monolayer 48 hours after treatment at two sites (2×10^6 cells per site) with macrophages derived from C57Bl/Ks mice immunized with heat-killed BCG.

monolayer. Two monolayers of each A/Jax, L-cell, and DBA/2 fibroblasts were employed. No significant destruction was apparent in any of the monolayers examined at 24, 48, and 60 hours (Fig. 1).

The capacity of cell-free extracts of immune cells to cause specific destruction of target cells was tested. Suspensions of well-washed macrophage-rich immune cell preparations, derived from C57Bl/Ks mice immunized with A/Jax antigens containing 100×10^6 cells per milliliter, were treated by the following methods: (i)

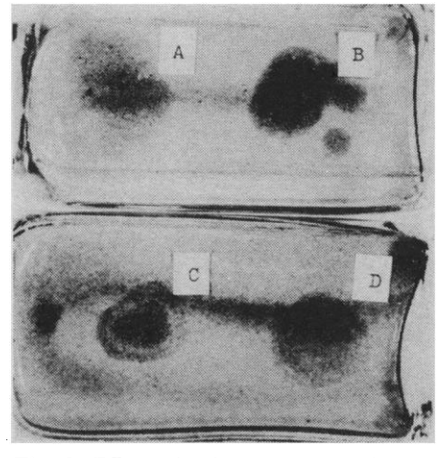

Fig. 2. Effect of prior treatment with inhibitor on the capacity of immune C57Bl/KS macrophages to adhere to L-cell target monolayers. (*A*) Nontreated control cells. (*B*) Sodium-azide–treated cells. (*C*) DNP-treated cells. (*D*) Sodium-fluoride–treated cells.

alternate freezing and thawing at −70°C and 37°C for 12 cycles, (ii) high-frequency sound treatment for 2 minutes at 1.3 amperes in a MSE ultrasonic disintegrator at 4°C, and (iii) homogenization in a Potter-Elvehjem grinder. The treated cell suspensions were centrifuged at 2100*g* for 15 minutes at 4°C, and the sediments were discarded. The extracts were stored at −70°C. Various dilutions of the extracts were added directly to cultures of the specific target cells, the A/Jax fibroblast and the L-cell, and to cultures of the nonspecific target cells, the C57Bl/Ks fibroblast and the HeLa cell. The monolayers were maintained on coverslips (1.0 by 2.0 cm) in screw-capped tubes (1.5 by 12 cm). At 24 and 48 hours the monolayers were stained by the May-Grunwald-Giemsa method and examined. The cell-free extract, derived from 100×10^6 immune peritoneal cells, commonly killed between 80 to 100 percent of both the specific and nonspecific target cells. The cells became granular and rounded, and exhibited nuclear pyknosis as early as 12 to 24 hours later. By 48 hours approximately 40 to 90 percent of the cells were detached from the glass.

Attempts were made to characterize the specific adherence factor associated with the immune-cell surface. Immune peritoneal cells were pooled and washed six times at 4°C, twice in 80 ml of Hanks balanced salt solution containing 1 percent calf serum and four times in physiologic saline, *p*H 7.0. The washing fluids were stored at −20°C for subsequent tests, and the macrophage-rich sediments were resuspended in 5.0 ml of physiologic saline. The suspensions, which contained approximately 200×10^6 cells per milliliter, were heated at 56°C for 1 hour. The heated cells were sedimented for 10 minutes at 2100*g* and the eluate was stored at −20°C. Control eluates were prepared from similar numbers of starch-stimulated peritoneal cells derived from nonimmune C57Bl/Ks mice. As a control, tests for the presence of specific adherence factors within the cytoplasm were made. Extracts of well-washed immune cells were made by alternate freezing and thawing and by treatment with high-frequency sound. Membranes were removed from these extracts by centrifugation at 30,000*g* for 5 hours.

The last washing fluid obtained before heating, the heat eluate, and the extracts were tested against A/Jax and C57Bl/Ks red blood cells for hemag-

glutinating antibodies by the polyvinylprolidone technique (*5*). The heat eluate showed a specific hemagglutinin titer of 2^6 to 2^7 against A/Jax red blood cells. Specific hemagglutinins were not detected in the eluate from control nonimmune cells or in the final washing fluid of immune cells or in membrane-free extracts of immune cells. Although the hemagglutinin was destroyed by exposure to 0.1*M* 2-mercaptoethanol for 60 minutes at 37°C, trials to establish its sedimentation behavior by sucrose-gradient techniques were not satisfactory.

The eluates were tested further by Terasaki's cytotoxicity test (*6*). Five different heat eluates from immune cells were capable of activating complement, as shown by their capacity to kill approximately 70 percent of normal A/Jax lymph-node cells in the presence of fresh rabbit serum.

The influence of metabolic inhibitors on the adherence and plaquing capacity of immune cells was tested. A suspension (0.5 ml) containing 2×10^6 of well-washed immune cells per milliliter was incubated for 2 hours at 37°C in 5.0 ml of Eagle's minimal essential medium plus high concentrations of cell inhibitors as follows: 0.1*M* sodium azide, 0.004*M* 2,4-dinitrophenol, 0.05*M* sodium fluoride, 10.0 μM actinomycin D, 100 μg of chloramphenicol per milliliter, and 200 μg of puromycin per milliliter. The cells were chilled to 4°C, sedimented, and suspended in 0.2 ml of Eagle's minimal essential medium. One drop of each treated cell suspension was then placed on a monolayer of A/Jax fibroblasts and a monolayer of L-cells. After 5 minutes, the monolayers were washed vigorously three times with a total of 30 ml of physiologic saline and stained by the plaque method (Fig. 2). Untreated control cells and inhibitor-treated cells adhered equally well to target cells. Specific adherence of the immune cell to the target cell apparently does not require a metabolically active immune cell. Actinomycin D and chloramphenicol-treated cells were washed before the plaque test. In some cases chloramphenicol was added to the medium (Fig. 3). Both actinomycin D and chloramphenicol blocked plaque formation. However, chloramphenicol-treated cells only remained inactive when initial treatment was supplemented by maintaining a concentration of 100 μg of chloramphenicol per milliliter of culture medium (Fig. 3, *A* and *B*). Whereas control immune peritoneal cells produced plaques (Fig. 3*D*), control starch-induced nonimmune C57B1/Ks peritoneal cells at the high dose of 2×10^6 cells failed to produce plaques (Fig. 3*C*).

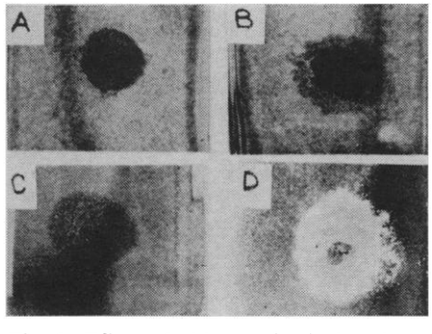

Fig. 3. Effect of chloramphenicol and actinomycin-D treatment on the capacity of immune C57B1/Ks macrophages to cause destruction of L-cell target monolayers after 48 hours. (*A*) Chloramphenicol-treated cells. (*B*) Actinomycin D–treated cells. (*C*) Normal peritoneal untreated cells. (*D*) Untreated immune cells (the central dark spot is a wax pencil mark).

With respect to the chance that complement or its components may participate in plaque formation, three possible sources of complement were considered: (i) "free" humoral complement, (ii) humoral complement which may become fixed to immune cells in vivo, and (iii) complement formed by either the immune cell or the target cell. The participation of free humoral complement appears to be ruled out by our observations that well-washed immune cells exhibit full plaquing activity in medium without serum or in medium containing heat-inactivated calf serum. The second possibility, that absorbed complement is present on the surface of the immune cell, was tested. Immune macrophages were unaffected by treatment with heat-inactivated rabbit antiserum to mouse tissue. No evidence was obtained that complement participates in plaque formation.

Thus, immune peritoneal macrophages derived from C57B1/Ks mice, immunized against A/Jax antigens, possess membrane-associated antibodies that may be responsible for specific contact adherence of the immune cell to the target cell. That specific adherence was not blocked by a variety of cell-inhibiting agents is consistent with the concept that adherence is passive and results solely from the reaction of a surface membrane antigen of the target cell with surface membrane-bound antibody of the immune cell.

The additional observation that actinomycin D– or chloramphenicol-treated immune cells were unable to destroy the target cells even though they adhered suggests that to form plaques the immune cell must have biosynthetic activity. In contrast some trials indicate that treatment of target cells with metabolic inhibitors does not affect plaque formation.

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Sulfur Mustard: Reaction with L-Cells Treated with 5-Fluorodeoxyuridine

Abstract. *After exposure to 5-fluorodeoxyuridine, L-cells are considerably more sensitive to the lethal effect of sulfur mustard than after they have been released from this block by addition of thymidine and allowed to proceed into the G2 phase of the division cycle. Nevertheless, for both populations, the amounts of mustard bound per cell and per nucleus (expressed as the amount of mustard per unit of protein) were the same. Likewise, the amounts of mustard bound per unit of DNA were the same for both populations.*

Cultured L-strain mouse cells were more sensitive to the lethal effect of nitrogen and sulfur mustards when the cells were synthesizing DNA than after they had entered the G2 period (*1*). This result was obtained with cell populations that had been treated with 5-fluorodeoxyuridine (FUDR) and then with thymidine, which releases the cells from the inhibitory effect of the FUDR. The cells then move in a wave successively through phases S and G2. Beyond this point the synchrony diminishes rapidly. Since the