hibition (*4*); on the other hand, it is an unsatisfactory way to investigate inhibition, because drug and enzyme are analyzed together, in vitro, which dilutes and distorts the original relation (*5*).

Keller (*6*) observed inhibition of carbonic anhydrase from bovine red cells by DDT (50 μg/ml) in vitro, whereas Wistrand [cited in (*4*)] found no inhibition of bovine enzyme, in amounts claimed by Keller. Anderson and March (*7*) were unable to detect any inhibition by DDT on insect carbonic anhydrase either in vivo or in vitro at concentrations up to 3550 μg/ml. We examined again, in vitro, whether DDT inhibits this enzyme. The matter is of much theoretical and practical importance, since carbonic anhydrase inhibitors clearly reduce the rate of calcium deposition in shell, both in birds and invertebrates [reviewed in (*4*)].

Carbonic anhydrase activity was analyzed by a colorimetric *p*H method (*8*) which measures the rate of hydration of carbon dioxide. Solutions of *p,p'*-DDT and *p,p'*-DDE were prepared in absolute ethanol or in DMF (*3*). The final concentration of DDT or DDE in the reaction vessel was 50 to 85 μg/ml in 16 percent ethanol or 5 percent DMF. Solutions were incubated with enzyme (human red cell) and solvent (with and without drug) up to 3 days at room temperature. No inhibition was observed. Concentrations greater than 50 μg/ml in the reaction mixture resulted in some precipitation of drug.

The effect of DDT on semipurified bovine carbonic anhydrase was examined by the method of Maetz (*9*), which had also been used by Keller (see above). This method measures the rate of dehydration of carbonic acid. A number of solvents were used; DMF (2.5 percent in final solution) yielded the most reliable data. Again Keller could not be confirmed; there was no inhibition at 100 μg/ml. Inhibition progressed from 37 to 88 percent as the concentration of DDT increased from 500 to 2000 μg/ml. In these experiments there was also some precipitation of drug in the reaction mixture. However, the degree of inhibition observed at 500 μg/ml is relatively small and suggests that DDT may not inhibit carbonic anhydrase effectively at the usual tissue concentrations found in organisms exposed to DDT in the environment. Further clarification is required because of solubility difficulties in the in vitro system. In addition, direct investigation of the inhibition of enzyme from the shell gland of birds is needed. However, no case has yet arisen in tissues of the vertebrate kingdom in which carbonic anhydrase inhibition by known drugs has been greater than that found against the enzyme in red cells (*4*).

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Tumor Immunity: Tumor Suppression in vivo Initiated by Soluble Products of Specifically Stimulated Lymphocytes

Abstract. *Supernatant fluids of specifically stimulated lymphocyte cultures were purified. Fractions containing migration inhibition factor when injected intradermally into strain-2 guinea pigs produced a reaction similar in appearance to delayed cutaneous hypersensitivity. There was an accumulation of mononuclear cells at the injection sites and the growth of syngeneic tumor grafts at the sites was suppressed.*

The immunologic rejection of tumors in syngeneic animals is mediated by specifically sensitized lymphoid cells (*1*). Delayed hypersensitivity has been associated with the rejection of some syngeneic hepatomas induced by diethylnitrosamine (*2*). However, hepatomas that do not provoke a delayed hypersensitivity reaction can be inhibited at the site of a delayed hypersensitivity reaction initiated by an unrelated antigen. Delayed hypersensitivity reactions consist of the specific recognition of an antigen by a relatively small number of sensitized lymphocytes followed by the accumulation of a relatively large number of mononuclear cells (*3*). We have found that macrophages from unimmunized animals, but not neutrophils or lymphocytes, can inhibit the growth of one of these tumors in vivo and in vitro (*4*). Cell-mediated tumor immunity, therefore, requires at least two distinct reactions: (i) specific interaction of sensitized lymphocytes and tumor cell antigen, and (ii) the local accumulation of mononuclear cells that prevent the growth of tumor cells at that site.

Lymphocytes incubated in vitro with the specific antigen to which they were sensitized produce substances that (i) inhibit the migration of macrophages from capillary tubes (*5*), (ii) are cytotoxic in vitro (*6*), (iii) are leukotactic (*7*), and (iv) can give skin reactions similar to delayed hypersensitivity (*8*). Tumor cell antigens have been shown to cause the release of macrophage migration inhibition factor (MIF) (*9*). We have been able to obtain inhibition of tumor growth at sites of inflammatory reactions produced by the intradermal injections of crude supernatants of specifically stimulated lymphocyte cultures (*10*). In this report we show that intradermal injection of tissue culture fluids containing MIF is followed by the accumulation of mononuclear cells and an inflammatory response at the site of injection. The growth of tumors at these sites is inhibited.

Age-matched, adult, Sewall-Wright NIH inbred strain-2 guinea pigs were used. Induction of primary hepatomas by the administration of diethylnitrosamine in the drinking water and the formation of an ascites variant have been described (*11*). Ascites cells from the sixth generation of a transplantable hepatoma (line 10) were prepared (*12*). In all experiments 10^6 tumor cells mixed with the appropriate reagent were injected intradermally in a volume of 0.1 ml. Each result given is the mean for three animals.

Inbred strain-2 guinea pigs were immunized by the injection of heat-killed *Mycobacterium tuberculosis* (0.1 ml,

Table 1. Inhibition of tumor growth in vivo by tissue culture fluids containing MIF.

Supernatant injected*	Delayed hypersensitivity skin reactions at 24 hours† (mm^2)	Size of intradermal tumor papule on day 14† (mm^2)
Control A	0	15 ± 1.0
Supernatant containing MIF	15.6 ± 2.5	0
Control D	0	9.3 ± 0.7
Medium 199	0	6.0 ± 1.0

* 10^7 tumor cells per milliliter of supernatant were incubated at 37°C for 10 minutes; 0.1 ml of the mixture was injected intradermally at each site. † Results are expressed as mean of the average radius squared (mm^2) ± standard error of the mean.

H37Rv strain 2 mg/ml in a 1 : 1 mixture of 0.15*M* NaCl and 15 percent Arlacel A in Bayol F) into each footpad and posterior nuchal area (five injections). Fourteen to 18 days later the animals were killed, the lymph nodes collected, and cell suspensions made as previously described (*13*). The cells were washed with Hanks balanced salt solution and resuspended in RPMI-1640 tissue culture medium to contain 3 to 4 × 10^6 viable cells per milliliter (1.2 to 1.6 × 10^7 per 4-ml of culture). The medium contained fresh frozen glutamine (15 mg/liter) and Ampicillin (100 μg/ml at 288 milliosmoles, *p*H 7.4). Cultures for MIF production contained PPD-S antigen (10 μg/ml) without preservation. Two control cultures were included: one without antigen (control A) and one with coccidioidin, an antigen unrelated to tubercle bacilli (control D). The supernatants, after 36 hours, were harvested, dialyzed against distilled water, lyophilized, and kept frozen at −70°C. Lyophilized samples were reconstituted with distilled water, and fractionated by gel filtration on Sephadex columns; the peak of biologic activity was located by testing the fractions for capillary migration inhibition (*13*). Sephadex G-75 was eluted with 0.01*M* borate buffer, *p*H 8, and the fraction having a molecular weight of 30,000 to 40,000 was collected and found to contain the MIF activity by the indirect assay. Pooled fractions of this peak were lyophilized and redissolved in 0.01*M* tris buffer, *p*H 8.7, and fractionated further by electrophoresis on polyacrylamide gel. With a 10 percent gel, separation *p*H 10.2, concentration *p*H 9.65, on a 1 by 20 cm column, electrophoresis was performed at 4°C (200 volts, 5 to 10 ma). The active MIF fraction migrated ahead of albumin. In the stained gel there was no visible band in the area containing active MIF. Active MIF could be eluted from the gel either by extraction of minced sections of gel in distilled water or by continued electrophoresis from individual sections into dialysis bags. The purified MIF fraction was dissolved in a volume of medium equal to the volume of the original supernatant fluid obtained from the lymphocyte cultures. MIF activity was present at this final concentration. Corresponding fractions of control A from Sephadex and polyacrylamide gels were used. An additional control consisting of supernatant fluid from unstimulated lymphocyte cultures and an amount of PPD-S equivalent to the maximal possible amount in the purified material had shown neither inflammatory nor tumor-inhibiting activity.

Fluids containing MIF and control fluids were injected intradermally. Skin reactions to fluids containing MIF were present at 3 to 4 hours and reached maximal size between 12 and 24 hours; there was no reaction to the intradermal injection of control fluids. Histologic sections of reaction sites taken at 24 hours revealed a typical picture of tuberculin hypersensitivity reaction. There was a preponderance (70 to 80 percent) of mononuclear cells. Supernatants incubated with tumor cells were injected intradermally. The results of this experiment are shown in Table 1. It can be seen that fluids containing MIF inhibit tumor growth and control fluids do not.

We next tested whether tumor rejection initiated by MIF at one intradermal site would affect tumor growth at another intradermal site. The design and results of this experiment are given in Table 2. It can be seen that a tumor adjacent to a rejected tumor was unaffected. This observation opposes the view that tumor rejection initiated by MIF was due to a systemic adjuvant effect.

It is possible that supernatants containing MIF exert a direct cytotoxic effect on tumor cells. In order to test this possibility, tumor cells were inoculated into skin sites where MIF or control supernatants had been injected 24 hours earlier. Tumor cell growth was inhibited (see Table 3) at sites where an inflammatory reaction was present before tumor cells were injected. This observation favors the view that tumor rejection was due to host cells rather than to a direct effect by MIF.

These experiments support the concept that animals immunized to a given tumor contain lymphocytes capable of specific immunologic interaction with that tumor and that following this interaction, the lymphocytes elaborate substances that cause the accumulation of mononuclear cells; these mononuclear cells are then responsible for the rejection of tumor grafts. Present evidence indicates that this activity of the mononuclear cells is immuno-

Table 2. Tumor growth in vivo at a site adjacent to MIF-mediated rejection of tumor cells.

Supernatant injected*	Growth of tumor cells inoculated in medium 199: size of papule on day 14†	Growth of tumor cells inoculated in MIF or control supernatants: size of papule on day 14†
Control A	19.9 ± 1.0	20 ± 2.3
Supernatant containing MIF	17.7 ± 3.0	0
Control D	16.2 ± 2.3	18.1 ± 1.2

* 10^7 tumor cells per milliliter of supernatant or medium 199 were incubated at 37°C for 10 minutes; 0.1 ml of the mixture was injected intradermally at each site. The distance between sites of tumor rejection and adjacent tumor sites was 1.5 to 2.0 cm. † Results are expressed as mean of the average radius squared (mm^2) ± standard error of the mean.

Table 3. Inhibition of tumor growth at sites of skin reactions to MIF.

Supernatant injected*	Size of skin reaction at 24 hours† (mm^2)	Size of tumor papule at 14 days† (mm^2)
Control A	0	7.1 ± 2.6
Supernatant containing MIF	16.4 ± 1.2	0.2 ± 0.2
Control D	0	5.9 ± 3.2

* 0.1 ml of each supernatant was injected intradermally 24 hours prior to the injection of 10^6 tumor cells in a volume of 0.1 ml into each site. † Results are expressed as mean of the average radius squared (mm^2) ± standard error of the mean.

logically nonspecific, and, therefore, tumor rejection by these cells does not require their specific recognition of tumor antigen.

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Vitamin D_3: Induction of Calcium-Binding Protein in Embryonic Chick Intestine in vitro

Abstract. *Induction of the synthesis of calcium-binding protein in chick embryonic intestine maintained in vitro was accomplished by simply adding vitamin D_3 to the culture medium. Accompanying the induction of this protein, there was enhanced radiocalcium uptake by the intestine. These observations represent the first demonstration of an in vitro physiological effect of vitamin D_3 on the calcium absorptive mechanism of the intestine.*

A fundamental action of vitamin D is the enhancement of intestinal calcium absorption. In recent years, this action has been linked to the vitamin D–induction of a calcium-binding protein (CaBP) in the intestine (*1*). The physicochemical properties (*2*) and cell localization (*3*) of CaBP, as well as numerous correlations between the intestinal concentration of CaBP and the calcium absorptive capacity of the intestine in various physiological, nutritional, and disturbed functional states (*4*), attest to a central role of this protein in the calcium transport mechanism.

As one approach in our continuing efforts to more fully define the role of CaBP in calcium transport, an in vitro organ culture system was developed which utilized embryonic chick intestine. It was found that vitamin D_3, when added to the culture medium, stimulated CaBP synthesis and enhanced the uptake of radiocalcium by the intestine. This marks the first demonstration of a direct action of vitamin D_3 on the intestine in vitro.

Table 1. Enhancement of ^{47}Ca uptake in cultured embryonic chick intestine by addition of vitamin D_3 to the medium. Duodenal tissue was cultured for 48 hours in the presence or absence of vitamin D_3 in the medium (400 I.U. per milliliter). The tissues were then transferred to a buffer solution (*10*) containing ^{47}Ca and incubated at 37°C for 30 minutes. After the tissues had been rinsed, blotted, and weighed, they were counted in a gamma spectrometer. The D_3 group value (mean of ten separate determinations) was significantly greater than the control value (ten determinations) at the 0.001 percent level (Student's *t*-test). The difference in ^{47}Ca uptake, though slight, was highly reproducible.

Vitamin D_3 in medium (400 I.U. per milliliter)	CaBP per gram of tissue (μg)	^{47}Ca uptake (percentage of dose per 100 mg of tissue)
−	0	13.14 ± 0.22
+	13.5	16.73 ± 0.67

In the general procedure, the duodenal loop from a 20-day-old chick embryo was excised and the pancreas was removed. It had previously been shown that there was no detectable CaBP in embryonic chick intestine; CaBP first appeared on the day of hatching (*5*). The duodenum was halved and slit longitudinally. The two segments were incubated in a 30-ml tissue culture flask (Falcon Plastics) at 37.5°C in 5 ml of fluid consisting of McCoy's 5A modified medium containing 30 percent fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and 100 units of nystatin per milliliter. After 48 hours, the segments were rinsed with 0.9 percent saline, drained on tissue paper, and weighed. They were then homogenized in 2 volumes of a tris buffer (0.0137*M* tris, 0.12*M* NaCl, and 4.74 m*M* KCl; *p*H 7.4), and the homogenates were centrifuged at 38,000*g* for 20 minutes.

The supernatants were analyzed for CaBP by radial immunodiffusion. In this method, uniform cylindrical reservoirs (10 mm in diameter by 3.2 mm deep) in a Lucite plate were filled with a buffered agar solution (1.5 percent agar, 7.5 percent glycine, and 0.02 percent thimerosal in barbital buffer, *p*H 8.6. After gelling, 10 μl of a highly specific rabbit antiserum to purified chick intestinal CaBP (*3*) were allowed to diffuse into each agar reservoir for at least 48 hours. Then a sample well was cut in the center of each agar reservoir, and 10 μl of the sample solution were dispensed into it. After a reaction period of from 24 to 48 hours, the presence of a visible precipitin ring around the central well confirmed the presence of CaBP. The diameters of the precipitin rings were measured microscopically. Plotting the log of the diameter against the log of the known concentration of purified CaBP yielded a linear relation over a range of 10 to 320 ng of CaBP. The absolute sensitivity of the method was 5 ng of CaBP; weaker reactions were enhanced by soaking the plate in a 0.0125 percent solution of cadmium acetate.

The results of one of a series of typical experiments in which crystalline vitamin D_3 (*6*) (Mann Research Laboratories, New York) was included in the culture medium are shown in Fig. 1. It is clear that vitamin D_3 [400 international units (I.U.) of vitamin D_3 per milliliter of medium] induced the formation of a substance