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

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References

1. L. J. Old, E. A. Boyse, D. A. Clarke, E. A. Carswell, *Ann. N.Y. Acad. Sci.* **101**, 80 (1962–63); G. Klein, H. O. Sjögren, E. Klein, K. E. Hellström, *Cancer Res.* **20**, 1561 (1960).
2. B. Zbar, H. T. Wepsic, H. J. Rapp, T. Boros, B. S. Kronman, W. H. Churchill, Jr., *J. Nat. Cancer Inst.* **43**, 833 (1969); B. Zbar, H. T. Wepsic, T. Boros, H. J. Rapp, *ibid.* **44**, 473 (1970).
3. R. T. McCluskey, B. Benacerraf, J. W. McCluskey, *J. Immunol.* **90**, 466 (1963).
4. B. Zbar, I. D. Bernstein, H. T. Wepsic, L. Stewart, T. Borsos, H. J. Rapp, in preparation; J. Oppenheim, B. Zbar, H. J. Rapp, *Proc. Nat. Acad. Sci. U.S.* **66**, 1119 (1970).
5. B. R. Bloom and B. Bennett, *Science* **153**, 80 (1966); B. Bennett and B. R. Bloom, *Proc. Nat. Acad. Sci. U.S.* **59**, 759 (1968); B. R. Bloom and B. Bennett, *Ann. N.Y. Acad. Sci.* **169**, 258 (1970); J. R. David, in *Mediators of Cellular Immunity*, M. Landy and H. S. Lawrence, Eds. (Academic Press, New York, 1969); J. R. David, *Proc. Nat. Acad. Sci. U.S.* **56**, 72 (1966); D. Thor, R. E. Jureziz, S. R. Veach, E. Miller, S. Dray, *Nature* **219**, 5155 (1968); D. Thor, in *Mediators of Cellular Immunity*, M. Landy and H. S. Lawrence, Eds. (Academic Press, New York, 1969).
6. N. H. Ruddle and B. H. Waksman, *Science* **157**, 1060 (1967); *J. Exp. Med.* **128**, 1267 (1968); G. A. Granger and W. P. Kolb, *J. Immunol.* **101**, 111 (1968); G. A. Granger, in *Mediators of Cellular Immunity*, M. Landy and H. S. Lawrence, Eds. (Academic Press, New York, 1969).
7. P. A. Ward, H. G. Remold, J. R. David, *Science* **163**, 1081 (1969).
8. B. Bennett and B. R. Bloom, *Proc. Nat. Acad. Sci. U.S.* **59**, 756 (1968); E. Pick, J. Krevci, K. Coch, J. L. Turk, *Immunology* **17**, 741 (1969); J. L. Turk, in *Mediators of Cellular Immunity*, M. Landy and H. S. Lawrence, Eds. (Academic Press, New York, 1969).
9. B. S. Kronman, H. T. Wepsic, W. H. Churchill, Jr., B. Zbar, T. Borsos, H. J. Rapp, *Science* **165**, 296 (1969); B. R. Bloom, B. Bennett, H. F. Oettgen, E. P. McLean, L. J. Old, *Proc. Nat. Acad. Sci. U.S.* **64**, 1176 (1969).
10. I. D. Bernstein, D. E. Thor, B. Zbar, H. J. Rapp, unpublished observations.
11. H. J. Rapp, W. H. Churchill, Jr., B. S. Kronman, R. T. Rolley, W. G. Hammond, T. Borsos, *J. Nat. Cancer Inst.* **41**, 1 (1968).
12. W. H. Churchill, Jr., H. J. Rapp, B. S. Kronman, T. Borsos, *ibid.*, p. 13.
13. D. E. Thor and S. Dray, *J. Immunol.* **101**, 51 (1968); D. E. Thor, in *In vitro Methods of Cellular Immunity*, B. Bloom and P. Glade, Eds. (Academic Press, New York, 1971).

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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

immunologically identical to chick intestinal CaBP. The reaction shown represents about 40 ng of CaBP or about 12 μg of CaBP per gram of cultured tissue. (By comparison, the chick duodenum after hatching contains from one to two times as much CaBP.) Further tests showed that as little as 25 I.U. of vitamin D_3 per milliliter of medium induced CaBP production (*7*), but detectable levels of CaBP were not usually seen prior to 48 hours incubation. That the substance producing the precipitin reaction was indeed identical to chick intestinal CaBP was verified by an immunoelectrophoretic procedure (*3*).

Two experimental procedures were used to determine the possible mechanism of CaBP synthesis in cultured embryonic duodenum in response to vitamin D_3. In the first, the antibiotics actinomycin D ($4.0 \times 10^{-6} M$), cycloheximide ($3.6 \times 10^{-5} M$), and puromycin ($5.3 \times 10^{-5} M$) were each separately incorporated in culture medium with or without added D_3 (400 I.U. per milliliter of medium). After 48 hours incubation, CaBP was present only in duodena incubated in D_3-containing medium without antibiotic. The inhibition of vitamin D_3–induced CaBP production by actinomycin D suggested that vitamin D–induction of CaBP is mediated through RNA synthesis, in support of in vivo findings (*8*). Inhibition of CaBP by cycloheximide and puromycin, which act at the ribosomal level, was to be expected from the actinomycin D effect. The conclusion to be drawn from this approach was that CaBP may have been produced in response to vitamin D_3–induction of specific RNA synthesis in this system.

A second approach utilized radioactive amino acid labeling of CaBP in vitro. In this method, embryonic duodenum was incubated in medium containing [^{3}H]leucine, with or without vitamin D_3 (400 I.U. per milliliter of medium). After 48 hours incubation, supernatants were prepared from the tissues and subjected to acrylamide gel electrophoresis (*3*). Advantage was taken of the fact that, in this system, CaBP migrates considerably faster than the other supernatant proteins. The gels were then assayed for radioactivity. The small peak of ^{3}H activity in the D_3 gel corresponding to the hatched area (Fig. 2, top) represents labeled CaBP. No such peak was observed in the control gel (Fig. 2, bottom).

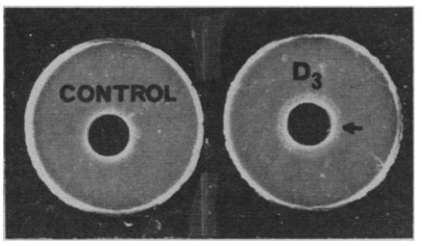

Fig. 1. Induction of CaBP in cultured embryonic chick intestine by addition of vitamin D_3 to the medium. The photograph illustrates two agar reservoirs in which antiserum to CaBP had been equilibrated. After addition of supernatant samples from intestines cultured in the presence (*D_3*) or absence (*control*) of vitamin D_3 in the medium (400 I.U. per milliliter), a visible precipitin ring (arrow) formed against the D_3 sample only.

Direct radial immunoassay was used to quantitate the exact amount of induced CaBP applied to the gel in the supernatant (378 ng), and the total protein applied (370 μg) was determined by the Lowry method (*9*). The specific activities could thus be calculated: for CaBP the value was 21×10^6 disintegrations per minute (dpm) per milligram of protein and, for all the rest of the proteins on the gel taken together, was 0.4×10^6 dpm per milligram of protein. Although the proteins other than CaBP may not have been uniformly labeled [in fact, other protein (or proteins) may have been as highly labeled as CaBP whether or not induced by vitamin D_3], this method does suggest that CaBP was synthesized de novo in response to D_3 in the culture medium.

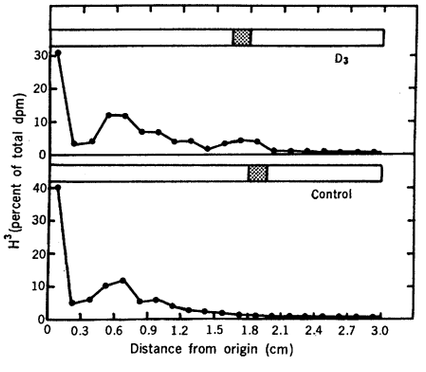

Fig. 2. Electrophoresis of supernatants prepared from embryonic chick duodenum cultured in the presence of [^{3}H]leucine. Duodenal tissue was incubated, as usual, in the presence or absence of vitamin D_3, in medium containing L-[4, 5-^{3}H]-leucine (9.4 μc/ml) (New England Nuclear). Supernatants of duodenal homogenates were then prepared. After approximately 1 μg of highly purified CaBP was added as a marker, the supernatants were subjected to electrophoresis on acrylamide gel discs; the discs were stained for protein visualization and were then sliced, dissolved, and counted (*15*). The hatched areas in the gel representations indicate the loci of the added CaBP on the gels; the solid circles, the percentage of the total ^{3}H activity on the gel in the particular 1.5-mm slice. The CaBP marker moved slightly faster in the control gel.

To determine if embryonic intestine, in which CaBP had been induced by vitamin D_3 in vitro, was capable of enhanced calcium uptake, duodenal tissue was incubated in the presence or absence of D_3 in the medium as before. The tissues were then incubated in a buffer solution (*10*) containing ^{47}Ca, and the total uptake was measured after 30 minutes. Table 1 shows that the duodena cultured in the presence of vitamin D_3 accumulated more ^{47}Ca than did the controls. Subsequent immunoassay revealed that CaBP was present in these duodena (13.5 μg of CaBP per gram of tissue) but not in the control duodena. Thus, two features of the in vivo vitamin D–stimulated intestine were duplicated in vitro: (i) CaBP induction and (ii) enhancement of calcium uptake.

The specificity of the induction of CaBP was tested by inclusion of different steroids in the culture medium at $2.6 \times 10^{-5} M$. Of the series tested, dihydrotachysterol-2 (Mann Research Laboratories), a vitamin D_2 derivative, was almost as potent on a molar basis as vitamin D_3 itself. 25-Hydroxycholecalciferol (HCC), a derivative of vitamin D_3 first isolated from pigs (*11*), was two to three times more potent than D_3. Cholesterol, 7-dehydrocholesterol, ergosterol, hydrocortisone, estradiol, and testosterone were ineffective. Although this list was not exhaustive, only steroids known to be biologically active in vivo were found to be effective in vitro.

In recent months it has been repeatedly suggested that vitamin D_3, prior to exerting its biological effect (*12*), may need to be metabolized in the liver to another form, 25-hydroxycholecalciferol. It was further suggested that the liver is the major if not the only site of HCC production. Liver hydroxylation was clearly not involved in the in vitro action of vitamin D_3 reported here. In fact, vitamin D_3 itself seemed to be able to initiate its known biological actions (*6*), namely, induction of CaBP and enhancement of calcium uptake by the intestine. However, the possibility of metabolism of vitamin D_3 to HCC in this culture

system was investigated. Radioactive [4-^{14}C]vitamin D_3 (N. V. Philips-Duphar, Amsterdam) was incorporated in the culture medium (80 I.U. of cold D_3 and 320 I.U. of [^{14}C]D_3, 0.4 μc/ml) and incubation was carried out as before. After 48 hours the entire contents of each flask were homogenized and a total lipid extraction performed (*13*). The lipid extract was concentrated by evaporation under a stream of N_2 and an aliquot was applied to a thin-layer plate (Eastman Chromagram), along with D_3 and HCC standards, and developed with 25 percent acetone in *n*-hexane. This system was capable of resolving D_3 ($R_F = 0.53$) and HCC ($R_F = 0.37$) cleanly. The chromatogram was cut into sections that were counted in a liquid scintillation counter. Approximately 2 percent of the total radioactivity remained at the origin, 95 percent migrated in the D_3 section, and the remainder was distributed uniformly from just after the origin through the HCC section at about 1 percent per section. Essentially similar results were obtained with extracts of the medium containing [^{14}C]-vitamin D_3 incubated with no tissue present. Thus, in this organ culture system, there appears to have been little or no conversion of vitamin D_3 to HCC during manifestation of vitamin D_3 action with the possible exception that further conversion of the HCC to some other form, which the chromatographic system was incapable of resolving (*14*), may have occurred. On the basis of these studies, it would seem necessary to consider the possibility that, at least, the vitamin D–responsive system in the intestinal mucosa exhibits a relative, rather than an absolute, specificity toward vitamin D–related sterols.

It is clear that the in vitro organ culture system described represents a unique opportunity to facilitate investigation of many aspects of the mechanism of action of vitamin D_3 on intestinal calcium transport.

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References and Notes

1. R. H. Wasserman and A. N. Taylor, *Science* **152**, 791 (1966).
2. R. H. Wasserman, R. A. Corradino, A. N. Taylor, *J. Biol. Chem.* **243**, 3978 (1968).
3. A. N. Taylor and R. H. Wasserman, *J. Histochem. Cytochem.* **18**, 107 (1970).
4. ———, *Fed. Proc.* **28**, 1834 (1969).
5. R. A. Corradino, A. N. Taylor, R. H. Wasserman, *ibid.*, p. 760. The normal chick embryonated egg contains vitamin D but, for unknown reasons, its intestine does not produce CaBP until after hatching. The in vitro results would suggest that the vitamin D present in ovo may not be accessible to the intestine or that there is some other physiologic mechanism maintaining the intestine in an unresponsive state until after hatching.
6. The purity of this preparation was checked by silicic acid thin-layer chromatography with the use of 25 percent acetone in hexane as developing solvent (vitamin D_3, $R_F = 0.53$; HCC, $R_F = 0.37$). When 10 percent phosphomolybdic acid in 95 percent ethanol was used as a spray reagent (detection limit, 0.25 μg of D_3 or HCC), no trace of HCC was found in a total of 2 mg of vitamin D_3 applied. Thus, in the culturing experiments, no more than 0.05 I.U. of HCC per milliliter could have been present in the medium. At least 5 I.U. of HCC per milliliter are necessary to induce a detectable amount of CaBP.
7. Although the concentrations of vitamin D_3 added to the medium were greater than blood concentrations of vitamin D in intact animals, the response of the embryonic intestine cannot be considered pharmacologic a priori, since the *effective* concentration of D_3 in the medium may be considerably less than the amount added because of insolubility, protein binding, or micelle formation. In addition, a typical physiologic response was obtained with as little as 25 I.U./ml (0.6 μg/ml) of D_3 which can hardly be considered a pharmacologic dose.
8. R. A. Corradino and R. H. Wasserman, *Arch. Biochem. Biophys.* **126**, 957 (1968).
9. O. H. Lowry, M. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
10. S. Hurwitz, H. C. Harrison, H. E. Harrison, *J. Nutr.* **91**, 319 (1967).
11. J. W. Blunt, H. F. DeLuca, H. K. Schnoes, *Biochemistry* **7**, 3317 (1968).
12. G. Ponchon, A. L. Kennan, H. F. DeLuca, *J. Clin. Invest.* **48**, 2032 (1969); G. Ponchon and H. F. DeLuca, *ibid.*, p. 1273; M. Horsting and H. F. DeLuca, *Biochem. Biophys. Res. Commun.* **36**, 251 (1969); H. F. DeLuca, *Fed. Proc.* **28**, 1678 (1969).
13. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
14. It should be noted that the data also do not exclude the possibility that D_3 might have been artifactually converted to HCC through some action of the medium itself, but even so the amount produced was near the borderline of CaBP-inducing capacity.
15. P. V. Tishler and C. J. Epstein, *Anal. Biochem.* **22**, 89 (1968).
16. Work was supported by NIH research grant AM-04652 and AEC contract AT(30-1)-4039. We thank Mrs. Frances Heistand for her technical assistance and Dr. John Babcock (Upjohn Co., Kalamazoo, Michigan) for repeated generous gifts of 25-hydroxycholecalciferol.

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Destruction of Mammalian Motor Nerve Terminals by Black Widow Spider Venom

Abstract. *Black widow spider venom selectively poisons motor nerve endings. A progressive and irreversible failure of neuromuscular transmission occurs in the cat. Electron microscopy of the poisoned nerve-muscle junction shows a sequence of motor nerve ending damage that culminates in disruption of the prejunctional membrane and loss of all organelles, including synaptic vesicles. The postjunctional membrane was morphologically unaffected. After complete poisoning, the contractile response to exogenous acetylcholine was severely impaired, an indication that the prejunctional site is chiefly involved in the contractile response produced by exogenous acetylcholine and that the pre- and postjunctional effects of acetylcholine were separated.*

The venom of the black widow spider (*Latrodectus mactans tredecimguttatus*) destroys amphibian motor nerve endings in what appears to be a highly specific action (*1*, *2*). After application of the venom to the frog nerve-muscle preparation in vitro, there was an enormous increase in the frequency of miniature end-plate potentials (MEPP), after which MEPP frequency declined to low levels. This increase and later decay were interpreted as reflecting a complete discharge of the transmitter stores (*1*). Neuromuscular transmission disappeared at a time when the MEPP frequency had reached a peak and nerve ending depolarization had occurred. Frog neuromuscular junctions that had been exposed to the spider venom were examined by electron microscopy (*2*) and showed a total absence of synaptic vesicles and other organelles; the terminal expansions were evacuated. There could be little doubt that these morphological changes were the cause of the venom-induced, complete and irreversible failure of neuromuscular transmission.

These findings raise the question of whether the spider venom would have a similar selective action on mammalian (cat) motor nerve endings. If there is selective destruction by the venom, then it should be possible to ascertain indirectly the role of the motor nerve endings in the contractile response that is evoked by the intra-arterial injection of acetylcholine (ACh) in innervated muscle in vivo. We had earlier found that intra-arterial ACh depolarizes the motor nerve endings and that the in vivo contractile response of the innervated muscle to ACh is largely a result of this action (*3*–*5*).

To establish the effect of the venom on neuromuscular transmission, the simple in situ nerve-muscle preparation of the cat was used (*3*). The cat was anesthetized with chloralose (80 mg/kg intravenously); the sciatic nerve was sectioned. The soleus nerve and muscle were exposed, and the leg was fixed in