an unlabeled pool of calcium such as intestinal contents. The serum calcium concentration for the 25-OHD_3–treated anephric rats was not significantly different from that of the control group.

The results in this report add to the body of evidence that vitamin D must be "activated" by hydroxylation first in the liver at C-25 (*14*) and then in the kidney (*1, 2*) at C-1 before it can produce its physiological functions in the intestine and bone. Frolik and DeLuca (*15*) have provided evidence that 1,25-$(OH)_2D_3$ is not metabolized further before it acts on intestine. Our results show that 1,25-$(OH)_2D_3$ or a further metabolite thereof is the metabolically active form of vitamin D responsible for bone calcium mobilization.

M. F. HOLICK, M. GARABEDIAN
H. F. DELUCA
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison 53706

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31 January 1972

Cytochalasin B Inhibits Lymphotoxin Production by Antigen-Stimulated Lymphocytes

Abstract. *Lymph node cells of rats sensitized with hen ovalbumin produced lymphotoxin after 6 to 12 hours of exposure to specific antigen. Lymphotoxin was assayed by its cytotoxicity for fibroblasts from syngeneic embryos during a 72-hour incubation. Cytochalasin B inhibited lymphotoxin production, as well as later DNA synthesis, at concentrations (0.1 to 5.0 micrograms per milliliter) comparable to those which affect microfilament function and cell motility in other systems, and this inhibition was reversible. Binding of antigen was not affected.*

Suitably primed T lymphocytes (thymus-dependent or thymus-processed cells), when stimulated by antigen, release a variety of physiologically active factors (*1*). Prominent among these are "lymphotoxin" (*2–4*), "proliferation inhibitory factor" (*5*), and an "inhibitor of DNA synthesis" (*6*). Release may also be triggered by nonspecific lymphocyte mitogens such as phytohemagglutinin and concanavalin A. Release precedes and is not necessarily correlated with increased synthesis of lymphocyte DNA and with blast transformation. The B lymphocytes (thymus-independent) can be stimulated by antigen (*7, 8*), antiserum to immunoglobulin (*9*), or mitogens such as lipopolysaccharide endotoxin (*10*) and appear to produce and release specific antibody (*8, 11*), but there is no information to show whether or not they also release active factors like those mentioned above.

The detailed mechanism of lymphocyte triggering has been extensively studied. The generally recognized sequence of RNA, protein, and DNA synthesis (*12–14*) is preceded by one or more steps initiated by the binding of antigen or mitogen to the cell membrane. The antigen-antibody aggregate or mitogen-receptor complex moves toward the uropod of the cell, and "cap formation" (*15–17*) and pinocytosis (*13, 16–18*) occur within the first minutes, followed by discharge of lysosomal hydrolases (*18, 19*) and gene activation (*13, 14*). The synthesis of new proteins, including lymphotoxin, begins 2 to 4 hours after addition of antigen.

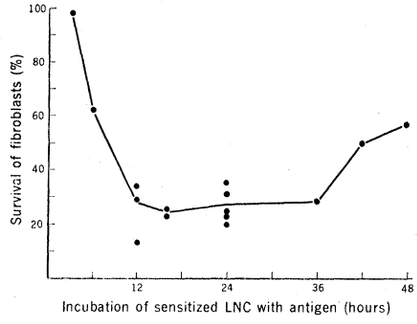

Fig. 1. Time course of lymphotoxin production. Sensitized lymph node cells (8×10^7) were incubated with ovalbumin (50 μg/ml) for varying lengths of time, and supernatants were tested. Results of four separate experiments are shown.

Taylor *et al.* (*17*) have shown that cap formation, pinocytic ingestion of antigen-antibody aggregates, and the selective disappearance of the latter (*17, 20*) are inhibited by cytochalasin B, a fungal product that interferes with microfilament function (*21, 22*) and inhibits many types of cell movement, including phagocytosis and pinocytosis (*17, 23*). It seemed of interest to determine the effect of this substance on cell triggering, as measured by the production and release of lymphotoxin (*2, 24*).

Young adult DA rats of both sexes were sensitized with 100 μg of hen ovalbumin (*25*) in complete Freund adjuvant, which was injected into the two hind footpads. Suspensions of sensitized lymph node cells (LNC) were prepared, 9 days after sensitization, from the inguinal and iliac lymph nodes. These and comparable normal LNC, usually 8 to 12×10^7, were incubated for various lengths of time with or without specific antigen (50 μg/ml) in 2 ml of "medium"—Ham's F10 medium (*26*) containing 10 percent fetal calf serum (*27*) plus penicillin (100 unit/ml) and streptomycin (100 μg/ml). Supernatants were cleared by centrifuging for 7 minutes at 800 rev/min and again for 15 minutes at 2400 rev/min and assayed for cytotoxic activity on monolayers of fibroblasts from syngeneic rat embryos (*2, 24*).

Fibroblasts giving a uniform size distribution after primary culture and three to five weekly transfers were plated in petri dishes measuring 60 by 15 mm (*28*), 1 to 2×10^5 cells in 4 ml of medium. After 48 hours, the medium was decanted and replaced with 2 ml of test supernatant plus 2 ml of fresh medium, and the cultures were incubated at 37°C in 5 percent CO_2 for 72 hours. At this time detached cells, shown earlier to consist almost entirely of dead cells, were removed by washing the culture twice with Hanks balanced salt solution. The remaining cells were treated with 2.5 ml of 0.25 percent Viokase solution (*27*), and the resulting homogenous suspension was counted in a Coulter model A electronic particle counter. All values shown in Table 1

Table 1. Reversible inhibition of lymphotoxin production by cytochalasin B. Sensitive or normal lymph node cells (18×10^7 in 3 ml) were incubated for 12 hours with or without ovalbumin (50 μg/ml) and cytochalasin B (5 μg/ml) or dimethyl sulfoxide (0.17 percent). After thorough washing, cells (12×10^7 in 2 ml) were reincubated for 16 hours with or without antigen. All supernatants from the first but not the second incubation were dialyzed for 12 hours before addition to the fibroblast monolayer (A, antigen; I, inhibitor; SF, surviving fibroblasts; DMSO, dimethyl sulfoxide; CB, cytochalasin B).

First incubation			Second incubation	
A	I	10^5 SF	A	10^5 SF
	Sensitive lymph node cells			
+	DMSO	2.6	0	4.3
+	DMSO	3.2	+	3.8
0	DMSO	7.6	+	3.2
+	CB	7.9	0	4.7
+	CB	7.9	+	4.1
0	CB	7.9	+	4.9
	Normal lymph node cells			
+	DMSO	7.9		
+	CB	7.9		

and Fig. 1 are averages of duplicate determinations. Cytotoxic activity, representing the presence of lymphotoxin, was detectable in supernatants of sensitive LNC incubated for 6 hours with ovalbumin and was maximal by 12 hours (Fig. 1). There was no subsequent increase in the apparent activity of lymphotoxin, and after 36 hours activity waned, perhaps because lymphotoxin was degraded enzymatically or inactivated by an inhibitor. Normal LNC did not produce lymphotoxin, nor did sensitive LNC incubated with heterologous protein antigens.

Cytochalasin B (*29*), 3 mg/ml in dimethyl sulfoxide (DMSO) (*30*), was diluted with medium, and 0.1 ml of the dilution was added to LNC cultures. Control cultures received DMSO diluted to the same extent. After incubation for 12 or 24 hours, the supernatants were routinely dialyzed for 12 hours at 4°C against three changes of Ham's F10 medium, while undialyzed control supernatants were held at 4°C. The removal of cytochalasin B by dialysis was essential because this substance inhibited fibroblast division and gave multinucleate cells, so that low fibroblast counts were false indicators of cytotoxicity. Lymphotoxin was not dialyzable. As shown in Table 1, lymphotoxin production was completely inhibited over a 12-hour period by cytochalasin B, 5 μg/ml. The differences in cytotoxicity shown were highly significant. In experiments not shown here, complete inhibition was also observed at cytochalasin B concentrations between 0.5 and 5 μg/ml, and partial inhibition was seen at 0.1 to 0.25 μg/ml. Once formed, lymphotoxin was not affected by incubation for 1 hour at room temperature with cytochalasin B and subsequent dialysis.

To test the reversibility of inhibition by cytochalasin B, 18×10^7 sensitive LNC in 3 ml were incubated for 12 hours with or without ovalbumin in the presence of cytochalasin B, 5 μg/ml. A 2-ml portion of the supernatant was dialyzed and assayed for cytotoxicity. The cells were washed three times in cold fresh medium, and 12×10^7 cells in 2 ml were incubated with or without additional antigen for a further 16 hours. These cells showed an essentially normal production of lymphotoxin (Table 1). Thus the effect of cytochalasin B is reversible and cannot be attributed to generalized cell damage. In fact, sensitive LNC incubated with ovalbumin in the presence of cytochalasin B showed higher viability, as measured by trypan blue exclusion, than did cells incubated without cytochalasin B.

To study a later event that normally follows specific antigenic stimulation, we examined DNA synthesis in similar sensitive LNC cultures (2×10^6 cells in 1 ml of medium) to which ovalbumin (25 μg/ml) and graded amounts of cytochalasin B were added. After 48 hours of incubation, tritiated thymidine (*31*) was added, 1.0 μc/ml, before a further 24 hours of incubation. Uptake of tritiated thymidine was determined as in earlier studies (*10, 32*). At cytochalasin B concentrations between 0.2 and 2.0 μg/ml, inhibition of DNA synthesis was proportional to the logarithm of the concentration (Fig. 2). This inhibition was completely reversible.

The observations reported here show that formation or release of lymphotoxin, part of the early protein synthesis occurring several hours after triggering of sensitized lymphocytes with specific antigen (*3*), is inhibited by cytochalasin B. Subsequent blast transformation, as measured by uptake of tritiated thymidine, is also inhibited, in agreement with the findings of Webster and Allison [cited in (*17*)]. Earlier work (*22*), showed in almost all instances a close correlation between movement presumably mediated by contraction of microfilaments and cell function; cytochalasin B produces morphologic changes in the former while inhibiting the latter. The concentrations at which lymphotoxin production and DNA synthesis

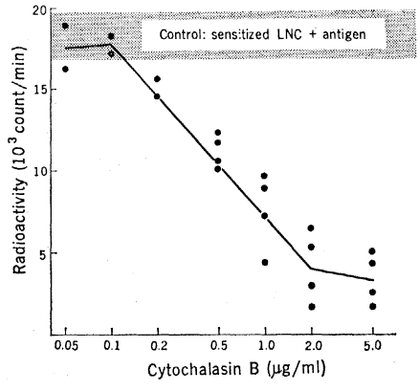

Fig. 2. Inhibition of incorporation of tritiated thymidine into sensitized lymph node cells. Cells (2×10^6) were incubated for 72 hours in the presence of ovalbumin (25 μg/ml) plus graded concentrations of cytochalasin B. A pulse of tritiated thymidine was given during the last 24 hours.

are inhibited and the reversibility of this inhibition suggest the similarity of our test system to others studied. We observed complete abolition of both lymphotoxin release and DNA synthesis at cytochalasin B concentrations greater than 0.5 μg/ml and partial inhibition at 0.1 to 0.25 μg/ml, concentrations comparable to those which affect spontaneous motility, chemotaxis, phagocytosis, and pinocytosis (*21–23, 33*). When we washed cells at the end of a 12-hour incubation with antigen and cytochalasin B, they formed lymphotoxin without further exposure to antigen. This establishes that cytochalasin B had no effect on binding of antigen as such but inhibited a later event. It may also imply that the antigen-antibody aggregates formed at the surface persist unaltered throughout this period. Possibly certain early events, such as the activation of adenylate cyclase (*34*), fail to go to completion or perhaps are irrelevant to microfilament contraction, pinocytosis, and the other early steps leading to gene activation and protein synthesis. Siskind and Thorbecke (*35*) have found that exposure to antigen at 4°C, followed by washing, triggers the cells for subsequent blast transformation. However, in their study, prolonged exposure (3 hours) resulted in desensitization ("tolerance") of the cells.

MASARU YOSHINAGA
BYRON H. WAKSMAN
STEPHEN E. MALAWISTA
Departments of Microbiology and *Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510*

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29. Imperial Chemical Industries, Ltd. Macclesfield, Chesire, England; molecular weight 479.
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36. Supported by PHS grants AI-06112, AI-06455, AM-10493, AM-5639, and AI-271; NIH international fellowship F05-TW-1708 held by M.Y. and research career development award AM-19864 held by S.E.M.; and by a grant from the Arthritis Foundation. We thank N. H. Ruddle for instruction in the use of her technique and D. Renshaw for technical assistance.

7 February 1972; revised 21 March 1972 ■

Auditory Induction: Perceptual Synthesis of Absent Sounds

Abstract. *Within certain auditory patterns, fainter sounds may be "heard" clearly when replaced by louder sounds having appropriate spectral compositions. This auditory induction of fainter by louder sounds can cancel the perceptual effects of masking. Phonemic restorations, which have been reported previously, appear to be a specialized application to speech of the much broader phenomenon of auditory induction. The rules governing auditory induction indicate that it helps maintain stable auditory perception in our frequently noisy environment.*

When a speech sound or syllable within a sentence is deleted and replaced by a louder extraneous sound, listeners believe they hear the excised portion and cannot detect which part of the sentence is missing (*1*). When we reported this ability to perceptually synthesize missing phonemes, we did not suspect that it could be considered as a special case of a much broader auditory phenomenon. Speech components are not the only types of sounds that can be "heard" when they are not present. There appear to be at least two other types of perceptual synthesis that can be grouped under the generic term of auditory induction (AI).

We discovered the second type of AI when we repeated without pauses sequences consisting of three intensity levels of the same sound. Thus, when a 2000-hz octave band noise was presented through headphones at three intensities (for example, 60, 70, and 80 db above 0.0002 μbar) with each successive level lasting for 300 msec, and the sequence was recycled without pause, then the faintest sound appeared to be on continuously, coexisting with each of the two louder sounds. This is a puzzling and seemingly paradoxical auditory effect, for were the fainter sound to remain on, it should fuse with the louder sounds of the same spectral characteristics. Nevertheless, all subjects (20 undergraduate students, 8 graduate students and staff) reported this AI whether or not they were aware of the physical nature of the auditory pattern and the paradoxical implications of perceiving the faintest sound as continuous. The order of the three sounds was not crucial (the position of the middle and loudest intensities could be interchanged), nor was the spectral composition of the sound. However, temporal contiguity was important, and 50 msec of silence between successive sounds prevented AI.

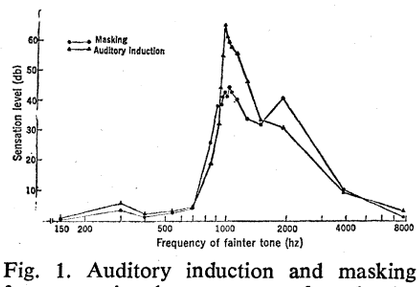

Fig. 1. Auditory induction and masking for tones in the presence of a louder 1000-hz tone presented at a sound-pressure level of 80 db. The values are the means for six subjects.

This type of AI involving spectrally identical sounds did not require that the repeated sequence consist of three intensities; 300-msec presentations of a 2000-hz octave band noise alternating between sound-pressure levels of 70 and 80 db resulted in illusory continuity of the fainter sound. Presenting other narrow noise bands, broad noise bands, and tones at alternating intensities also produced AI. These observations suggested the possibility that alternating the intensity of spectrally different sounds (for example, tones of two frequencies, or a tone and a noise) might give rise to a third type of AI having illusory persistence for durations comparable to those of the other two. There have been reports that this type of illusory continuity exists at rapid alternation rates (*2*). In these experiments, the illusory persistence of the fainter of two qualitatively different sounds was limited to about 5 to 90 msec, with