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29. For Gauss and Gilbert groups, the uniform component has been set to the value determined by least-squares analysis of Brunhes and Matuyama groups (see text). Fisher (*21*) precision parameter (kappa) and circular standard deviation (CSD) were estimated using a numerical simulation that adds Gaussian noise to inclination values represented in each data set. The variance of the Gaussian noise is set equal to the square of the root-mean-squares (rms) residual determined in the least-squares fit to the corresponding data. These rms values range from 4.6° (for the Brunhes chron) to 6.9° (for the Gilbert chron). After adding noise, the best fitting dipole axis was recomputed by the least-squares method. This was repeated 1000 times and the results were averaged with standard Fisher statistics. From this average, we calculated the precision parameter and CSD and presume that these estimates roughly match those of the true population from which the best fitting dipole axis position was drawn.
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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Kills Immature Thymocytes by Ca^{2+}-Mediated Endonuclease Activation

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Suspensions of thymocytes from young rats were incubated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which resulted in a sustained increase in cytosolic free Ca^{2+} concentration followed by DNA fragmentation and loss of cell viability. Both the Ca^{2+} increase and DNA fragmentation were prevented in cells treated with the inhibitor of protein synthesis, cycloheximide, and DNA fragmentation and cell killing were not detected when cells were incubated in a "Ca^{2+}-free" medium or pretreated with high concentrations of the calcium probe, quin-2 tetraacetoxymethyl ester. These results indicate that TCDD can kill immature thymocytes by initiating a suicide process similar to that previously described for glucocorticoid hormones.

GLUCOCORTICOID HORMONES, cytotoxic T lymphocytes, and natural killer cells all activate a process in target cells that is known as "apoptosis" or "programmed cell death" (*1*). Characteristic of this process is widespread chromatin condensation, which has been related to the stimulation of an endogenous endonuclease that cleaves host chromatin into oligonucleosome-length fragments (*2*). Recently we showed that an early, sustained increase in cytosolic Ca^{2+} concentration was critically involved in the activation of DNA fragmentation in glucocorticoid-treated thymocytes (*3*). A protein synthesized in response to glucocorticoid stimulation appeared to elevate the cytosolic Ca^{2+} level in thymocytes by facilitating the influx of extracellular Ca^{2+}.

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent of a group of halogenated, aromatic hydrocarbons that occur as industrial by-products and environmental contaminants. Despite extensive investigation, the mechanisms involved in TCDD-induced toxicity remain unknown. TCDD causes thymic atrophy in laboratory animals by depletion of the small, immature cortical cells of the thymus (*4*). This pattern of cell death resembles that observed in the thymus in response to glucocorticoid treatment (*5*). Glucocorticoids and TCDD also have overlapping or indistinguishable effects in many other target organs. For example, both are potent inducers of specific isozymes of cytochrome P-450 in the liver (*6*), and both suppress B lymphocyte differentiation (*7*). In addition, the structural properties of the TCDD receptor are remarkably similar to those of the glucocorticoid receptor (*8*). Since the purified glucocorticoid receptor binds selectively to regions of cloned DNA whose transcription is regulated by glucocorticoids in vivo, and the nucleotide sequences essential for receptor-DNA interaction are functional in vivo as hormone-dependent regulatory or transcriptional enhancer elements (*9*), the similar binding properties of the TCDD and glucocorticoid receptors could imply that they activate similar genetic targets, causing similar effects.

To determine whether TCDD could exert its influence on the thymus through endonuclease activation, we investigated the effects of TCDD on several parameters characteristic of apoptosis in immature thymocytes. Chromatin condensation is an early and characteristic morphological change occurring in apoptotic cells (*1*). Glucocorticoid hormones induce this morphology change in thymocytes soon after treatment. Chromatin condensation has been detected only in cells exhibiting extensive DNA fragmentation, and it is a property of apoptotic thymocytes that allows them to be separated from normal cells on the basis of bouyant density (*10*). Extensive plasma membrane and nuclear envelope blebbing is another morphological marker for programmed cell death that typically accompanies glucocorticoid-induced chromatin condensation (*1*). We found that after 1 hour of incubation the

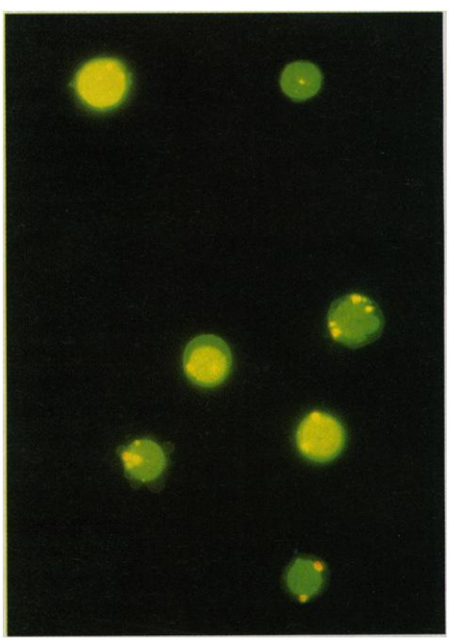

Fig. 1. Chromatin condensation in thymocytes exposed TCDD. Suspensions of thymocytes (50×10^6 per milliliter) from 3-week-old (50- to 55-g) male Sprague-Dawley rats were incubated in rotating, round-bottomed flasks in Krebs-Henseleit buffer, *p*H 7.2, supplemented with 10 m*M* Hepes, 15 m*M* glucose, and 1% bovine serum albumin, at 37°C under an atmosphere of 95% O_2:5% CO_2, in the presence of 10 n*M* TCDD, dissolved in dimethyl sulfoxide, for 2 hours. Samples were then stained with acridine orange (5 μl of a solution containing 100 μg/ml plus 95 μl of thymocyte suspension). Cells were then visualized with a fluorescence microscope. Magnification: ×1000.

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majority of thymocytes treated with 10 n*M* TCDD contained condensed nuclei; a photograph of a characteristic population of such TCDD-treated thymocytes is shown in Fig. 1. TCDD also induced widespread plasma membrane and nuclear envelope blebbing. The TCDD-induced morphological changes were similar to those observed in thymocytes exposed to the glucocorticoid methylprednisolone.

The most characteristic biochemical feature of the activation of the suicide process in glucocorticoid-treated thymocytes is the fragmentation of host DNA into oligonucleosome-length fragments (*2*). To determine whether TCDD treatment produced a similar pattern of chromatin cleavage, thymocyte suspensions were treated with 10 n*M* TCDD, and samples of DNA were isolated and analyzed by gel electrophoresis for comparison of relative fragment sizes (Fig. 2). TCDD treatment resulted in a 50 ± 14% (mean ± SEM, $n = 6$) loss of thymocyte viability by 5 hours (as measured by trypan blue uptake), compared with a 5 ± 3% (mean ± SEM, $n = 12$) loss of viability in control cells. The TCDD-induced loss of thymocyte viability was preceded by extensive hydrolysis of DNA. Electrophoresis of DNA from TCDD-treated thymocytes revealed a digestion pattern indistinguishable from that observed in methylprednisolone-treated thymocytes, suggesting that the activation of a common mechanism may be responsible for the effects on DNA of both compounds.

To obtain a profile of DNA fragmentation in TCDD-treated thymocytes, we measured the time course of endonuclease activation in cells treated with 100 n*M* TCDD (Fig. 3). In such cells we witnessed early fragmentation of chromatin, which proceeded at a rate that was considerably faster than that induced by glucocorticoids at up to 100-fold higher concentrations (*2*), suggesting that TCDD is a more potent activator of thymocyte suicide than glucocorticoid hormones are. TCDD-induced chromatin cleavage preceded measurable loss of cell viability by several hours, showing that DNA fragmentation was not the consequence of cell death.

Inhibitors of macromolecular synthesis, such as cycloheximide or actinomycin D (*3, 10*), block glucocorticoid-induced endonuclease activation in thymocytes. To determine whether TCDD-induced endonuclease activation was also dependent on protein synthesis, we treated thymocytes with cycloheximide before addition of TCDD. In control cells, cycloheximide administration caused slight stimulation of endonuclease activity relative to that observed in untreated cells, whereas TCDD-induced DNA fragmentation was almost completely inhibited in cycloheximide-treated thymocytes (Fig. 3). Thus both TCDD and glucocorticoid induction of thymocyte apoptosis required target cell protein synthesis.

To investigate the effect of TCDD treatment on the cytosolic Ca^{2+} concentration, we loaded thymocytes with a relatively low concentration of the fluorescent, Ca^{2+}-sensitive dye quin-2 tetraacetoxymethyl ester (quin-2 AM) and then exposed the cells to increasing concentrations of TCDD. The hydrophobic, esterified form of quin-2, which is quin-2 AM, readily diffuses through the plasma membrane into the cytosol, where esterases convert it to its polar form, trapping it within the cell. Treatment of thymocytes with 1 to 100 n*M* TCDD caused concentration-dependent increases in cytosolic Ca^{2+} level that were sustained for at least 2 hours of incubation (Fig. 4). Since pretreatment of cells with either actinomycin D or cycloheximide prevented the glucocorticoid-induced elevation of cytosolic Ca^{2+} concentration in thymocytes (*3*), we investigated the effect of cycloheximide treatment on the cytosolic Ca^{2+} increase elicited by the highest concentration of TCDD tested. In control cells, cycloheximide caused an early, transient increase in cytosolic Ca^{2+} concentration that returned to the basal level by approximately 90 min. Identical results were obtained from cycloheximide-treated cells subsequently exposed to TCDD (Fig. 4). Thus cycloheximide blocked the sustained increase in cytosolic Ca^{2+} concentration caused by TCDD, suggesting that the TCDD-induced elevation of

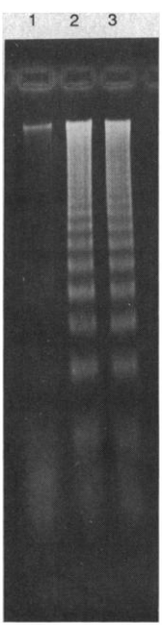

Fig. 2. Agarose gel electrophoresis of DNA fragments isolated from methylprednisolone- and TCDD-treated thymocytes. Thymocytes (50×10^6 per milliliter) were incubated in Krebs-Henseleit medium in the presence or absence of methylprednisolone (10 μ*M*) or TCDD (10 n*M*). Aliquots of 2 ml were lysed in 3 ml of a buffer containing 5 m*M* tris-HCl, 20 m*M* EDTA, 0.5% Triton X-100, *p*H 8.0, for 15 min on ice before centrifugation for 20 min at 27,000*g* to separate intact chromatin (pellet) from DNA fragments (supernate) (*10*). Samples were extracted sequentially with equal volumes of phenol, phenol:chloroform 1:1, and chloroform, precipitated in 67% ethanol, 0.17*M* NaCl at −20°C for 18 hours, resuspended in 10 m*M* tris-HCl, 1 m*M* EDTA, 0.5% SDS, *p*H 8.0, prior to electrophoresis for 2 hours at 60 V in 1.4% agarose gels. DNA was visualized by ultraviolet fluorescence after staining the gels with ethidium bromide. Lane 1, control; lane 2, 10 μ*M* methylprednisolone; and lane 3, 10 n*M* TCDD.

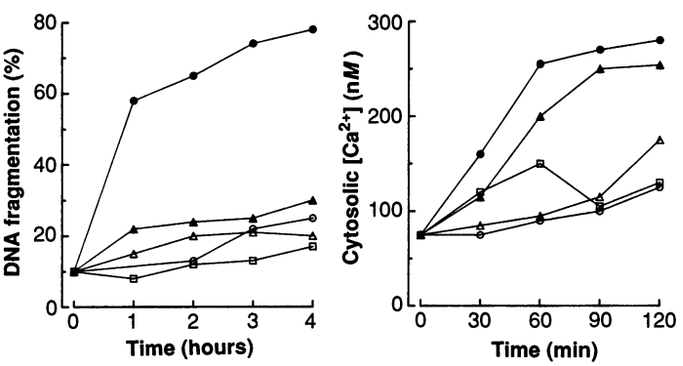

Fig. 3 (left). Inhibition of TCDD-stimulated endonuclease activity by cycloheximide, quin-2, and EGTA. Thymocytes (50×10^6 cells per milliliter) were incubated in the Krebs-Henseleit medium with or without 100 n*M* TCDD or with TCDD plus 10 μ*M* cycloheximide, 50 μ*M* quin-2 AM, or 8 m*M* EGTA. Thymocytes treated with 50 μ*M* quin-2 AM contained 1 m*M* hydrolyzed quin-2 1 hour after addition and 0.7 m*M* hydrolyzed quin-2 2 hours after addition. In no case did treatment of control cells with inhibitors affect viability, as measured by trypan blue uptake. At appropriate time points, 2-ml samples were lysed and centrifuged (see Fig. 1). Pellets were resuspended in 5 ml of a buffer containing 10 m*M* tris-HCl and 1 m*M* EDTA, *p*H 8.0. Pellet and supernate samples were assayed for DNA content with the diphenylamine reagent (*19*). Treatments: (○) control, (●) TCDD, (▲) TCDD plus cycloheximide, (△) TCDD plus quin-2 AM, and (□) TCDD plus EGTA. These results of one experiment were typical of four replicates. **Fig. 4 (right).** Effect of TCDD on cytosolic Ca^{2+} concentration in thymocytes. Thymocytes (50×10^6 per milliliter) were preincubated in the Krebs-Henseleit medium for 15 min. Cells were loaded with 5 μ*M* quin-2 AM for at least 15 min, which resulted in an intracellular quin-2 content of 100 μ*M*. Thymocytes were then incubated in the presence or absence of TCDD or TCDD plus 10 μ*M* cycloheximide. Aliquots of 1 ml were taken at appropriate time points, washed free of extracellular quin-2 AM and of hydrolyzed quin-2 by centrifugation for 30 s at 1500*g* in a microcentrifuge, and resuspended in 2 ml of Krebs-Henseleit medium (37°C) before measurement of cytosolic Ca^{2+} concentration (*20*). The addition of $MnCl_2$ to cells after washing resulted in no change in basal fluorescence intensity, showing that washing eliminated all extracellular quin-2. Measurements were discontinued after 120 min because of loss of sensitivity due to leakage of quin-2. Treatments: (○), control, (●) 100 n*M* TCDD, (▲) 10 n*M* TCDD, (△) 1 n*M* TCDD, and (□) 100 n*M* TCDD plus 10 μ*M* cycloheximide. These results of one experiment were typical of four replicates.

cytosolic Ca^{2+} concentration in thymocytes is dependent on protein synthesis, in a fashion analogous to that observed upon treatment of thymocytes with glucocorticoids. This observation also suggests that TCDD-induced protein synthesis in thymocytes most likely results in the production of a factor similar, or identical, to that synthesized in glucocorticoid-treated thymocytes, which facilitates the influx of extracellular Ca^{2+} (*3*).

To determine whether chromatin fragmentation in TCDD-treated thymocytes was dependent on the observed increase in cytosolic Ca^{2+} concentration, we loaded thymocytes with a concentration of quin-2 AM sufficient to buffer intracellular Ca^{2+} changes (*11*) before measurement of TCDD-induced DNA fragmentation. These cells had resting Ca^{2+} levels that were roughly 50% (40 ± 10 n*M*, mean ± SEM, $n = 4$) of those found in control cells (90 ± 25 n*M*, mean ± SEM, $n = 4$), and the increase in Ca^{2+} concentration due to TCDD exposure was inhibited for at least 2 hours (80 ± 20 n*M*, mean ± SEM, $n = 4$). Moreover, pretreatment of thymocytes with a high concentration of quin-2 AM completely blocked activation of the endogenous endonuclease by TCDD administration (Fig. 3). The Ca^{2+} ionophore A23187 can restore endonuclease activity in glucocorticoid-treated, quin-2–buffered thymocytes, showing that quin-2 does not interfere with endonuclease function directly (*3*). Thus, as is true with glucocorticoids, TCDD activation of thymocyte chromatin fragmentation is dependent on a sustained increase in cytosolic Ca^{2+} concentration.

To investigate the origin of the TCDD-induced Ca^{2+} increase in thymocytes, we tested the effect of incubation of thymocytes in a "Ca^{2+}-free" medium (*12*) on TCDD-induced DNA fragmentation. In control cells, incubation in such a medium had no effect on basal cytosolic Ca^{2+} level for up to 3 hours. However, incubation of thymocytes in this Ca^{2+}-free medium resulted in complete inhibition of TCDD-induced chromatin cleavage (Fig. 3). Thus, TCDD, like glucocorticoids, seems to activate a process that results in the influx of Ca^{2+} into treated thymocytes. This event appears to result in endonuclease activation and subsequent cell death.

Previous work (*13*) has provided evidence for direct action of TCDD on epithelial cells of the thymus, resulting in a reduction of mitogen-inducible proliferative capacity in cocultured thymocytes, but TCDD treatment did not result in thymocyte cytotoxicity. Although altered maturation of thymocytes may be a significant result of prolonged TCDD action, it does not explain the "scattered necrosis" of the small cortical cells caused by TCDD within the thymus (*4*). Moreover, the small cortical cells appear to be the least sensitive subpopulation of thymocytes to mitogen-induced proliferation (*14*). Thus, the depletion of cortical thymocytes observed in TCDD-treated animals is probably due to activation of the suicide process described here, rather than to inhibition of thymocyte proliferation.

Our findings stand in contrast to a previous study, in which TCDD was found to have no toxic effect on 23 different cell lines derived from tissues sensitive to TCDD in vivo (*15*), suggesting that a cell-cell interaction or humoral factor absent from in vitro systems is required for TCDD toxicity. The most likely explanation for the discrepancy is that we used only freshly isolated thymocytes from 3-week-old animals in this study, which have been shown to be the most sensitive cell type to glucocorticoid-induced apoptosis (*5*), whereas the previous study utilized cells and cell types that are relatively insensitive to the process. However, we cannot exclude the possibility that the contrasting results may be due to differences in incubation techniques [primary culture (*13, 15*) versus cell suspensions (present study)], or to the presence of a humoral factor that mediated TCDD toxicity in our system that was absent from the previous studies.

Several lines of evidence argue against a "shared receptor" mechanism for glucocorticoid- and TCDD-induced chromatin cleavage. Sensitivity to TCDD-induced thymic atrophy segregates with the *Ah* locus (*16*), which strongly suggests that the effects of TCDD on the cells of the thymus are mediated through the Ah receptor. Furthermore, the rapid and extensive fragmentation of thymocyte chromatin observed in this study was elicited by TCDD at concentrations two orders of magnitude lower than those at which glucocorticoids are effective. On the other hand, high concentrations of glucocorticoids and TCDD relative to in vitro receptor binding affinity constants (*8*) are required for the induction of thymocyte apoptosis. The reason for this is unclear, although there appears to be a correlation between TCDD and glucocorticoid receptor binding affinities and the dose of TCDD or glucocorticoid required to activate thymocyte apoptosis, TCDD being more potent in each case. We propose, therefore, that TCDD induction of thymocyte suicide is mediated by a specific receptor for TCDD (presumably the Ah receptor), although further studies are required to exclude the possibility that TCDD could bind to thymocyte glucocorticoid receptors that have a higher affinity for TCDD than for glucocorticoids themselves.

The results presented here outline a novel mechanism of action for a toxic agent, and may in part explain the effects of TCDD on the immunological status of exposed animals. However, an explanation for why TCDD produces thymic atrophy is insufficient to explain the lethality of the agent. Possibly of more general relevance to the study of how TCDD kills laboratory animals is the finding that TCDD can affect the cytosolic Ca^{2+} level in target cells. A sustained increase in cytosolic Ca^{2+} concentration has been linked to the toxicity of a variety of agents (*17*). Evidence for the generality of TCDD alteration of intracellular Ca^{2+} homeostasis is supported by the recent finding that TCDD appears to cause an increased intracellular Ca^{2+} concentration in the heart (*18*), although the mechanism by which TCDD increases the cardiac Ca^{2+} level remains to be elucidated. Further studies will be required to determine whether the results here represent a general mechanism of TCDD action.

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A *Mycobacterium leprae*–Specific Human T Cell Epitope Cross-Reactive with an HLA-DR2 Peptide

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***Mycobacterium leprae* induces T cell reactivity and protective immunity in the majority of exposed individuals, but the minority that develop leprosy exhibit various types of immunopathology. Thus, the definition of epitopes on *M. leprae* antigens that are recognized by T cells from different individuals might result in the development of an effective vaccine against leprosy. A sequence from the 65-kD protein of this organism was recognized by two HLA-DR2–restricted, *M. leprae*–specific helper T cell clones that were derived from a tuberculoid leprosy patient. Synthetic peptides were used to define this epitope as Leu-Gln-Ala-Ala-Pro-Ala-Leu-Asp-Lys-Leu. A similar peptide that was derived from the third hypervariable region of the HLA-DR2 chain, Glu-Gln-Ala-Arg-Ala-Ala-Val-Asp-Thr-Tyr, also activated the same clones. The unexpected cross-reactivity of this *M. leprae*–specific DR2-restricted T cell epitope with a DR2 peptide may have to be considered in the design of subunit vaccines against leprosy.**

LEPROSY CONTINUES TO BE AN IMportant endemic disease in most countries of Asia, Africa, and Latin America, with 10 to 12 million estimated cases worldwide (*1*). A problem is that resistance develops to the widely used drug dapsone. A promising alternative to this and other more expensive combined drug therapies is the possibility of an effective vaccine against leprosy. Most people can respond to infection by *Mycobacterium leprae* with both a humoral and cellular immune response. The latter, however, seems to control the outcome of an infection. Victims of lepromatous leprosy, the most severe form of the disease, have a specific anergy to *M. leprae* in their cellular immune response, which allows unlimited multiplication and spread of this bacillus. However, victims of tuberculoid leprosy suffer from an enhanced cellular immune response which can result in nerve destruction (*1*). It will be important for vaccine development to understand this difference in response. We are, therefore, defining specific interactions of *M. leprae* with the immune system and have begun to catalog *M. leprae* T cell epitopes. One important *M. leprae* antigen is the 65-kD protein, a prominent mycobacterial protein similar in sequence to the heat shock proteins (*2*). This highly immunogenic protein contains an *M. leprae*–specific B cell epitope (*3*) and a number of T cell epitopes (*4, 5*).

The HLA-DR2–restricted (*6*) *M. leprae* reactive T cell clone 2F10 appeared to be nearly *M. leprae*–specific. At low mycobacterial lysate concentrations 2F10 reacted only with Dharmendra lepromin and armadillo-derived *M. leprae* (Fig. 1); at higher concentrations it reacted weakly with *M. vaccae*. It did not react with 18 other species of mycobacteria, including *M. tuberculosis* (*7*). This particular clone was selected because it proliferated in the presence of the 65-kD protein of *M. leprae* [produced in *Escherichia coli* (*8*)], but not the *M. tuberculosis* Bacille Calmette Guerin (BCG) 65-kD protein [produced in *E. coli* (*9*)] (Table 1). Thus this clone recognizes an *M. leprae*–specific epitope on the 65-kD protein.

To further define the epitope, we synthesized peptides from *M. leprae*–specific regions of the 65-kD sequence. The only positive peptide, peptide 17, contains residues NH_2-terminal to, and including the epitope defined by, the *M. leprae*–specific mouse monoclonal antibody (MAb) IIIE9 (*3*) (Table 2). Peptide 17 almost fully activates clone 2F10 at a concentration of 0.35 n*M*, a half-stimulating concentration considerably lower than the 1 to 100 μ*M* range of many stimulatory peptides for helper T cells (*10, 11*). The activation of this clone is inhibited by the mouse MAb IIIE9, which recognizes a linear *M. leprae*-specific epitope on the 65-kD protein (*3, 12*), but not by F67-13, another mouse MAb to the 65-kD protein (*3*). Thus, peptide-induced T cell proliferation is inhibited by specific antibody, as has been previously demonstrated in a few cases (*13–15*).

We used different synthetic peptides to determine the exact location of the epitope recognized by 2F10 (Fig. 2). Data from

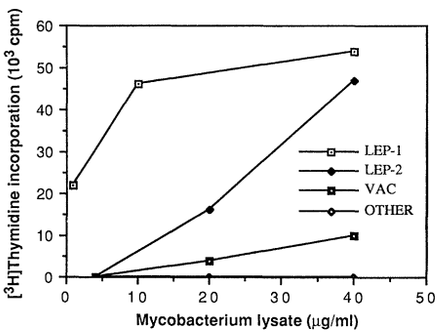

Fig. 1. Mycobacterial species-specificity of the helper T cell clone 2F10. Mycobacterial antigens were presented via autologous antigen presenting cells to 2F10. Details of the proliferation assay are as described (*7*). LEP-1 is Dharmendra lepromin, LEP-2 is armadillo-derived *M. leprae*, VAC is *M. vaccae*. "Other" represents mycobacteria giving no response: *M. kansasii*, *M. tuberculosis*, *M. avium*, *M. africanum*; *M. scrofulaceum*; *M. tuberculosis* strains H37Rv and H37Ra; *M. nonchromogenicum*; *M. gordonae*; *M. bovis*; *M. fortuitum*; *M. bovis BCG*; *M. duvalii*; *M. smegmatis*; *M. lepraemurium*; *M. lufu*; *M. avium/intracellulare*; and armadillo-derived aspecific mycobacteria.

Table 1. Specificity of the helper T cell clones 2F10 and 2B6. Antigen presentation, cloning, expansion, and proliferation assays were performed as described (*7*). Clones 2F10 or 2B6 (1×10^4 cells) were incubated with autologous mononuclear cells (5×10^4). Medium or bacterial lysates (10 μg/ml) were included in the assay. Each experiment was performed on three independent replicate cultures.

Addition to assay*	[^{3}H]Thymidine incorporation (cpm)	
	2F10	2B6
Medium	303 ± 139	277 ± 55
Y4178	26,907 ± 807	24,087 ± 2,408
1402	500 ± 80	132 ± 446
pEX II	392 ± 50	150 ± 22
CD75	53,343 ± 1,600	82,830 ± 3,313

*Y4178, *E. coli* expressing the gene for the *M. leprae* 65-kD protein (*8*); 1402, *E. coli* expressing the gene for the *M. bovis* BCG 65-kD protein (*9*); pEX II, *E. coli* expressing the control plasmid; CD75, armadillo-derived *M. leprae*.

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