Institution	: Schepen Eye Research Institute
Address	: 20 Staniford Street, Boston, MA 02114
Project title	: *In vitro* assay for hapten-specific priming of human T lymphocytes
Date	: Dec.29, 2000
Principal Investigator	: J. Wayne Streilein, M.D.
Co-investigator	: Sharmila Masli, Ph.D.
Grant number	: RO3 OH03650

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List of Abbreviations

DLN	Draining Lymph Node
DNFB	Dinitrofluorobenzene
CrO	Croton Oil
CH	Contact hypersensitivity
ACD	Allergic contact dermatitis
ICD	Irritant contact dermatitis
CSD	Conventional sensitizing dose (0.5% or 185 μg for DNFB)
OSD	Optimum sensitizing dose (0.004% or 1.5 μg for DNFB)
IFNγ	Interferon gamma
APC	Antigen Presenting Cell
Mϕ	Macrophage

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Abstract

Allergic and irritant contact dermatitis (ACD and ICD) are common inflammatory skin conditions that result after exposure of the cutaneous surface to certain environmental agents (contact sensitizers or haptens). Lack of clear understanding of immunopathogenesis of ACD so far has remained the major hindrance in achieving cure or prevention of these disorders. Effort towards prevention of ACD include identification of environmental agents that induce contact hypersensitivity (CH) response as well as individuals that are susceptible for such a response. Although it is known that certain universal contact sensitizing agents are chemically reactive low molecular weight compounds that can derivatize proteins upon contact and become immunogenic, it is not known why some contact sensitizers do not induce CH in all the individuals. Also, currently available means for the identification of contact allergens can not descriminate between allergens and irritants. In vitro assays designed to identify contact sensitizers so far have met with limited success. These assays have relied on the proliferative responses of T cells which is a predominant characteristic of a CH response besides inflammation. However, T cell proliferation has remained an inconsistent indicator for distinguishing haptens from irritants. Based on the distinct sensitizing properties of haptens as compared to irritants we propose to develop an in vitro assay that may help identify potential contact sensitizers and irritants as well as individuals susceptible to developing ACD or ICD. We have so far successfully demonstrated the possibility of generating hapten-specific sensitized T cells in vitro cultures in which autologous naive T cells that are exposed to hapten derivatized cultured PBMC for 5 days. In order to generate sensitized T cells that closely resemble those found in vivo we now plan to compare effector functions of T cells primed in vivo and in vitro. These comparisons will include phenotype of the T cells proliferating in a hapten-specific manner, their cytokine profile and hapten-specific cytotoxicity. Attempts will be made to compare and contrast T cell responses to various haptens and irritants. Thus this project can help (a) refine an in vitro assay for future application in clinical laboratory for screening various potential haptens and irritants as well as susceptible individuals and (b) provide better understanding of cellular events that lead to allergic Vs. irritant contact dermatitis.

The purpose of this project is to develop and implement an in vitro assay system that would allow identification and distinction of haptens that cause contact hypersensitivity as against irritants. Furthermore, such an assay could provide a useful tool to study mechanisms underlying a contact hypersensitivity response as well as screen individuals for any exposure to potentially irritating or allergenic agents.

Significant Findings

(1) It is possible to replace APC-driven stimulus used to detect sensitized T cell population with one mediated by monoclonal anti-CD3 antibody.

Antigen-primed T cells can be distinguished from naïve T cells in vitro by their differential proliferative response when stimulated with monocloncal anti-CD3 antibodies.

(2) Five days after epicutaneous application of a hapten (DNFB), DLN of mice contain anti-CD3 responsive T cell population that proliferates vigorously and secretes significantly higher levels of IFN-γ, whereas such a T cell population is not detected in the DLN of mice exposed epicutaneously to an irritant (Croton oil).

(3) However, within 24 hr of epicutaneous application of a conventional dose of an irritant (croton oil) DLN of mice also contain anti-CD3 responsive T cells that proliferate in a manner similar to hapten-sensitized T cells harvested 5 days after hapten exposure. However, such a T cell population is not detectable in the DLN of mice exposed to a conventional dose of hapten (DNFB) within 24 hr.

(4) Croton oil exhibits stronger irritant property, as measured by ear swelling response within 24 hr, as compared to DNFB.

(5) Unlike the conventional dose (0.8%), lowest/optimum dose (0.0004%) of Croton oil that causes irritant effect fails to induce anti-CD3-responsive T cells in DLN of mice within 24 hr.

(6) Similar to conventional dose (0.5%), optimum sensitizing dose (0.004%) of DNFB induces anti-CD3-responsive T cells that secrete significant levels of IFNγ in DLN of mice, 5 days after the initial exposure.

Conclusions: Haptens can be distinguished from irritants because only exposure to the former generates anti-CD3 responsive T cells in draining lymph nodes 5 days after exposure.

(7) When naive T cells are incubated with cultured Langerhans cells (without any hapten) for 5 days, activated T cells are generated that respond by vigorous proliferation to anti-CD3 mediated stimulus and also secrete IFNγ.

(8) Hapten-derivatized APC when cultured with naive T cells secrete significantly higher levels of IL-12 as compared to similarly cultured irritant-treated APCs.

Conclusions: The critical difference between haptens and irritants – with regard to distinguishing their differing effects on T cells – rests within APCs. The data strongly suggest that the amount of IL-12 generated by APC in response to haptens, especially in the presence of hapten-specific T cells, is vastly greater than that generated by APCs in response to irritants.

Usefulness of findings

Our results have demonstrated that T cells in lymph nodes draining skin sites painted in vivo with haptens or irritants can be stimulated to proliferate in vitro by anti-CD3 antibodies. However, when the T cells are tested at 5 days after epicutaneous application, only cells from hapten-painted donors proliferate and secrete IFN-g in response to anti-CD3. , whether hapten-sensitized or activated by an irritant, exhibit similar proliferative properties in response to anti-CD3 stimulus and can secrete IFNγ. These data are consistent with the hypothesis that, under the conditions of these experiments, only primed "memory" hapten-specific T cells are detectable at 5 days after hapten sensitization. Unfortunately, no similar distinction between hapten and irritant was detected when Langerhans cells, treated with hapten or irritant, were used to stimulate unprimed (naïve) T cells in vitro. Upon repeated testing, experiments attempting to use in vitro sensitization of T cells as an assay to distinguish hapten from irritant, proved to be inconsistent. We therefore turned our attention to the effects of irritants and haptens on antigen presenting cells. Our results are encouraging in this direction since we detected a significant difference in the ability of APCs to produce IL-12 (a key cytokine known to be involved in the induction of CH) when exposed to a hapten as opposed to an irritant. This result was further amplified if the treated APCs were then cultured with naïve T cells. We believe that these results indicate that strategies to distinguish haptens from irritants in vitro should be directed at their differing effects on APCS, rather than examining the eventual consequence on T cells.

1. Detection of T cells primed in vivo using modified protocol :

As described in our previous progress report, we modified our original protocol for an in vitro assay. In this assay APC-driven stimulus was utilized to determine proliferative response of T cells that were exposed to hapten either in vitro or in vivo. However, such an approach caused higher background proliferation limiting detecton of proliferation of hapten-sensitized T cells in some cases. Thus inconsistency in the autoreactivity of human T cells led us to work with our original murine system which formed the basis of the in vitro sensitization assay using human PBMC (1). In our modified protocol we replaced APC-driven stimulus with the one mediated by monoclonal anti-CD3 antibody. Applying this modification it was possible to distinguish T cells exposed in vivo to a hapten (DNFB) vs an irritant (croton oil). According to the original in vivo sensitizing protocol, Balb/c mice were sensitized by epicutaneous application of DNFB (0.5% in acetone) or croton oil (0.8% in acetone). Five days later, T cells were isolated from DLN of mice. These cells were then cultured in serum-free RPMI1640 (supplemented with 1:500 dilution of ITS+ mixture and 1% BSA) along with anti-CD3 antibody (2C11, 1:500) in a 96-well plate ($4x10^5$/well) in a final volume of 200 µl. Cultures were incubated at 37^0C, with 5% CO_2, for 48 hr and pulsed for final 6 hr with ^{3}H-thymidine. As evident from the proliferation profile (Figure 1), these results demonstrate that five days after the initial exposure to a hapten DLN of mice contained T cells that respond to anti-CD3 stimulus by vigorous proliferation whereas, such a T cell population was not found in DLN of croton oil exposed mice.

2. Comparison of irritant effect induced by croton oil vs DNFB :

Irritant effect of croton oil was compared with that of DNFB on the basis of ear swelling response induced by various concentrations of these two chemicals. Balb/c mice were exposed to various concentrations of either croton oil or DNFB by epicutaneous application to ear pinnae. Ear thickness was measured with an engineer's micrometer (Mitutoyo, Japan) prior to exposure (0 hr) and at various time intervals within 24 hr. Change in ear thickness at these time points compared to 0 hr was recorded as ear swelling response. It was noted that ear swelling response caused as an irritant effect was transient and undetectable beyond 24 hr after the initial exposure to either of the above chemicals. Figure 2a shows comparison of ear swelling responses at different time intervals after the initial exposure to croton oil. Based on significant response detected, 12 hr interval was determined to be the optimum time to assess an irritant effect.

As we have shown in our earlier report (2), optimum sensitizing dose for DNFB was determined to be 0.004% (1.5 µg). That is this dose of DNFB was the lowest tested dose capable of inducing sensitized T cells and therefore CH in mice. To determine optimum irritant dose various concentrations of croton oil and DNFB

were tested for their irritant effect. Optimum (lowest) dose of croton oil that induced significant ear swelling response was 0.0004% (Figure 2b) whereas concentrations of DNFB below 0.2% (dose generally used for ear challenge in CH assay) failed to induce significant irritant effect (Figure 2c).

3. Comparison of in vitro characteristics of T cells exposed to a hapten vs an irritant :

Proliferative response to anti-CD3 antibody of T cells from DLN of mice exposed to conventional doses of DNFB (0.5%) vs croton oil (0.8%) was assessed within 24 hr of exposure. This response was found to be contradictory to that described above at the 5 days interval. As shown in Figure 3, significantly higher proliferation was detected in T cells isolated from croton oil exposed mice as compared to those harvested from DNFB exposed mice. That is, within 24 hr DLN of animals exposed to croton oil contained T cells that responded to an anti-CD3 stimulus by vigorous proliferation whereas, such a population was missing in DLN of DNFB-exposed animals.

This result led us to test proliferative responses of T cells after exposure of mice to an optimum irritant dose of croton oil or an optimum sensitizing dose (which does not exhibit irritant effect) of DNFB. Within 24 hr of exposure neither chemical induced an anti-CD3-responsive population in DLN of mice. Even so, such a population of proliferative T cells was still detectable when lymph nodes were removed at 5 days after exposure to OSD of DNFB (Figure 4). These results demonstrated that DNFB sensitized T cells known to be present in mice exposed to OSD of hapten, 5 days after the initial exposure, are anti-CD3-responsive.
Furthermore, when IFNγ secretion was assessed by ELISA in culture supernatants collected from these proliferation assays, signficantly increased levels were detected only when T cells were isolated 5 days after exposure to either OSD or CSD of DNFB compared to croton oil (Figure 5).

These observations suggest that activated T cells with lowered threshold for activation (proliferative response and the ability to secrete IFNγ) are induced by irritants as well as haptens. However, such cells are maintained for at least five days only in DLN of mice exposed to a hapten – not an irritant. In other words, our results are consistent with the known transient nature of an irritant effect (in this case on T cells) as compared to the long lasting effect of haptens on specific T cells, such as are found in the contact hypersensitivity response.

Thus hapten sensitized T cells and T cells activated by an irritant both exhibit similar proliferative properties in response to anti-CD3 stimulus and can secrete IFNγ. Therefore proliferative profile in combination with IFNγ secreting ability does not allow distinction between inflammatory or hapten-specific immune response immediately after the exposure to a chemical but is rather applicable at a later time interval. Application of modified protocol to our original in vitro T cell sensitization protocol using cultured Langerhans cells failed to distinguish hapten-

sensitized T cells from non-specifically activated T cells in the primary cultures (Figure 6).

4. Ability of antigen presenting cells to produce IL-12 after exposure to DNFB vs croton oil :

Due to overlap in characteristics, detectable in vitro, of T cells exposed to DNFB and croton oil we shifted our focus from effector cells to antigen presenting cells. We have reported before that the ability of APCs to secrete IL-12 does play a role in determining whether and what type of effector cells are generated (3). Also, recently in a project funded independently, to determine genetic markers that would allow identification of contact sensitizing potential of various chemicals, we have detected various genes up- or down-regulated in APCs that can or cannot induce CH responses when injected into naïve mice. For this project, differential display analysis was applied to compare the genetic profile of two murine macrophage hybridomas (mf #59 and #63) (4). It was noted that many of the differentially regulated genes are known to be involved in the regulation of a cytokine IL-12. This observation along with the reported critical role of IL-12 in the induction of CH response (5) led us to test the ability of APCs to secrete IL-12 after exposure to DNFB vs croton oil.

Murine mϕ #59 (constitutively capable of inducing CH) was used as an APC. These cells were derivatized with DNFB as described before (1) or treated with sub-toxic concentrations of croton oil in a similar manner. These treated APCs (10^5/well) were then co-cultured in a 96 well plate with syngeneic naive T cells (0.5×10^5/well), in a serum-free RPMI1640 medium in a total volume of 200 µl. Culture supernatants were collected at 48 and 120 hr time intervals and tested by ELISA for the levels of IL-12. Figure 7 shows the IL-12 levels detected in these cultures. Significant difference was detected between the DNFB vs croton oil treated APC containing cultures, at a time interval of 120 hr.

These results suggest that haptens and irritants differ in their ability to influence APC which, in turn, dictates the properties of effector cells that eventually are responsible for triggering the immune response. Further investigation and comparison at the level of signal transduction within APCs in response to a hapten vs an irritant will reveal more details of the underlying mechanisms that may result into differences in immune response. Thus, we feel strongly that analysis of differentially expressed genes in APCs expose to haptens and irritants will reveal differences that can readily be exploited in vitro to distinguish these two important classes of compounds that cause allergic contact and irritant dermatitis.

References

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Publications

The results of these experiments have been submitted in an abstract to NORA 2001 symposium. Completion of additional experiments will allow their incorporation into a manuscript.

Figures

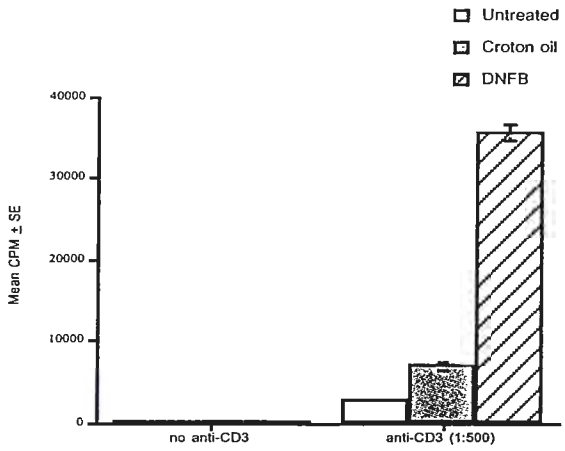


Figure 1 : Proliferative response 48 hr after anti-CD3-mediated stimulation of T cells isolated f rom DLN of mice treated five days previously with DNFB (0.5%) or Croton oil (0.8%).

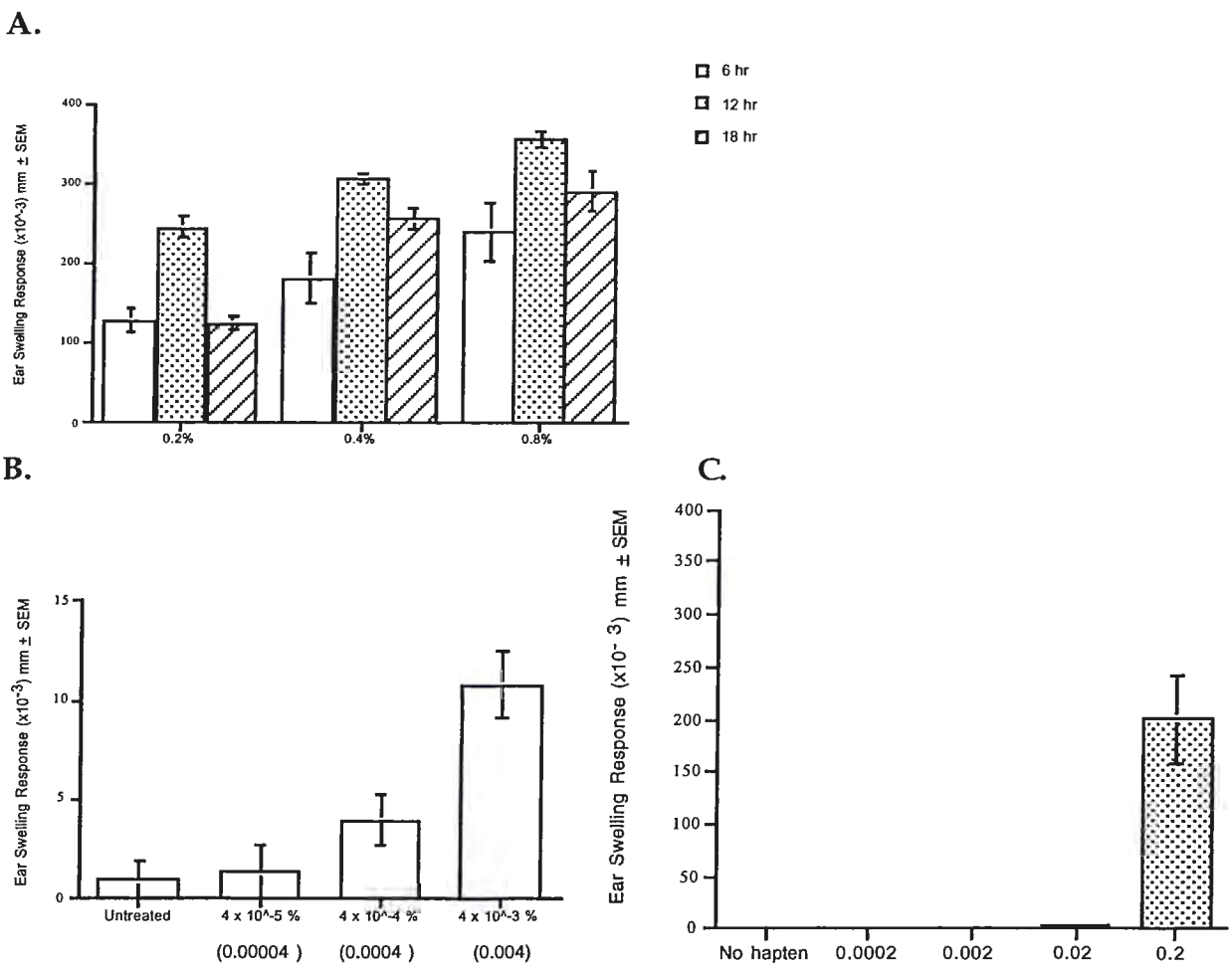


Figure 2 : Irritant effect of Croton oil or DNFB, measured as ear swelling response. BALB/c mice were exposed to various concentrations of Croton oil. Ear swelling responses were measured at 6, 12 and 18 hr Intervals thereafter (A). Irritant effect at 12 hr interval was compared with different concentrations of Croton oil (B) and DNFB (C).

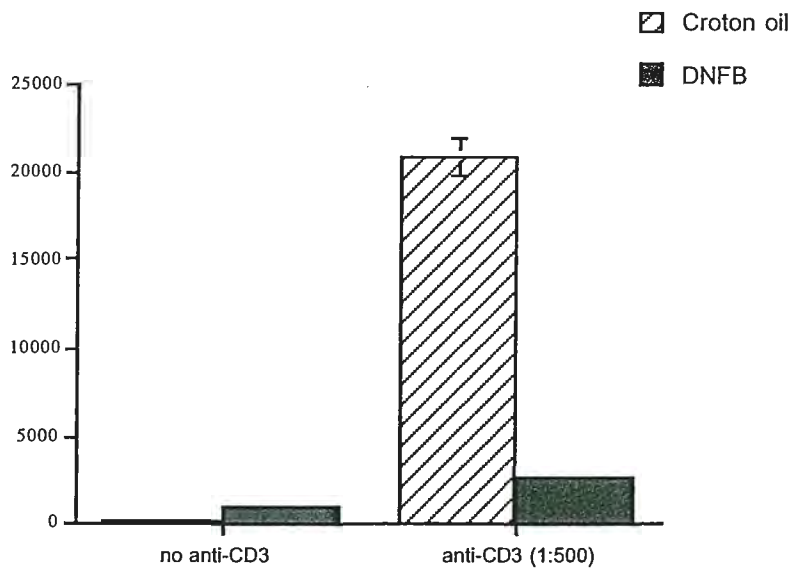


Figure 3 : **Proliferative response 48 hr after anti-CD3-mediated stimulation of T cells isolated from DLN of mice exposed 24 hrs previously to croton oil or DNFB.**

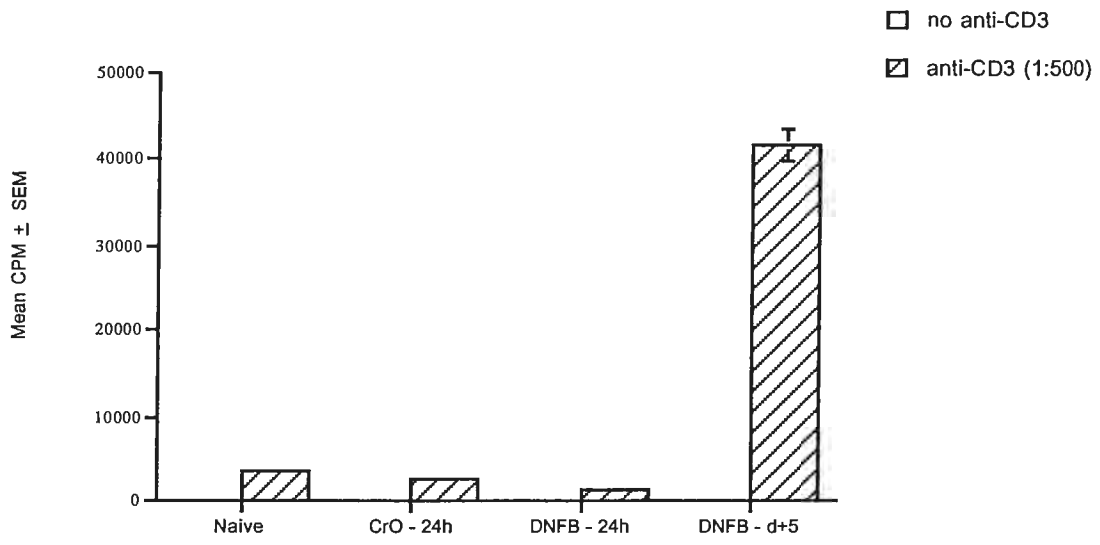
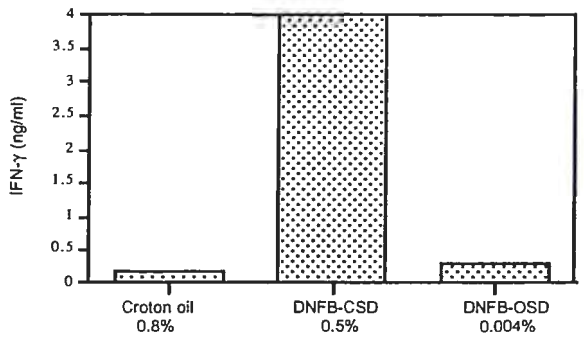


Figure 4 : **Comaparison of proliferative responses of T cells, harvested from DLN of mice exposed to Croton oil or DNFB (for 24 hr or 5 days) to anti-CD3 mediated stimulus.**

A.



B.

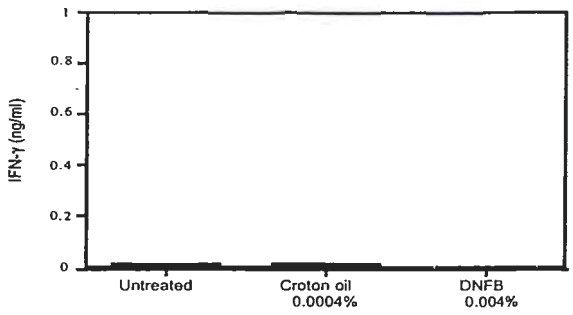
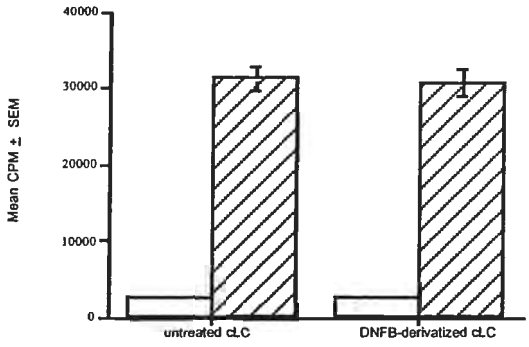


Figure 5 : IFNγ secreted by T cells in culture supernatants, 48 hr after anti-CD3 mediated stimulus. T cells were harvested from DLN of BALB/c mice that were exposed to DNFB or croton oil 5 days (A) or 24 hr (B) previously.

A.



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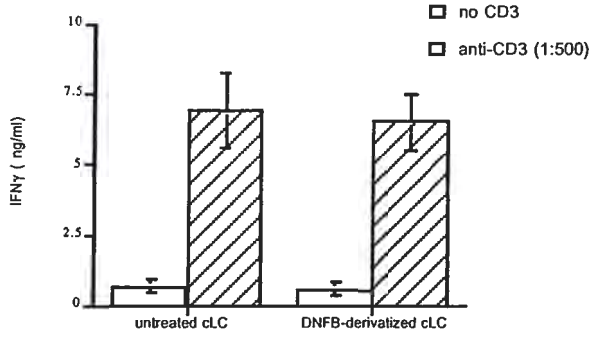

Figure 6 : Proliferation (A) and IFNγ secretion (B) by T cells stimulated for 48 hr with anti-CD3. T cells were harvested from primary cultures containing untreated or DNFB derivatized cultured Langerhans cells as APCs (in vitro assay as described in the scientific report)

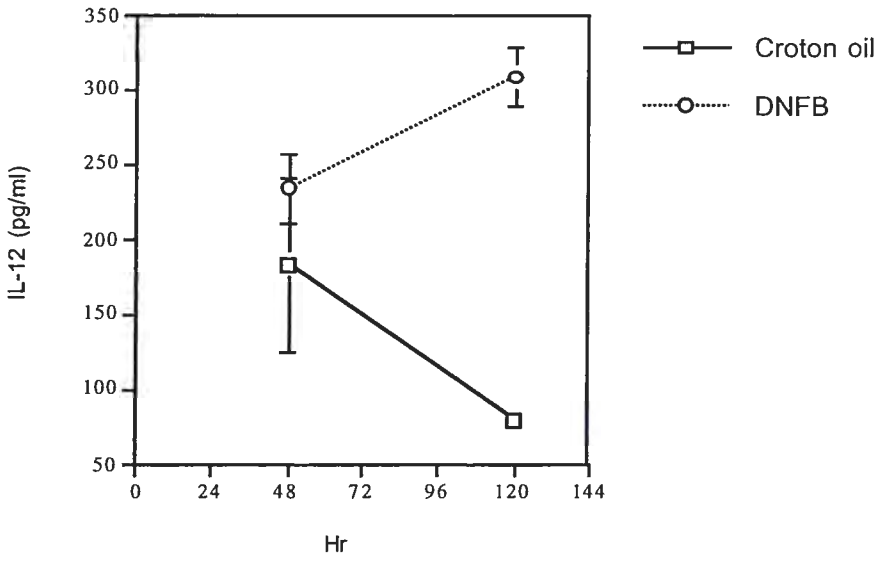

Figure 7 : **IL-12 detected in the supernatants of cultures containing naive syngeneic T cells stimulated with DNFB or croton oil treated macrophage hybridoma #59, at different time intervals.**

Equipment inventory

No equipment was acquired under this grant (RO3 OH03650).

Final Invention Statement

No inventions were conceived under this grant (RO3 OH03650)

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service
Centers for Disease Control
and Prevention (CDC)

Memorandum

Date: May 9, 2001

From: Roy M. Fleming, Sc.D., Director, Research Grants Program RMF
Office of Extramural Programs, NIOSH, D30

Subject: Final Report Submitted for Entry into NTIS for Grant 5 R03 OH003650-02.

To: William D. Bennett
Data Systems Team, Information Resources Branch, EID, NIOSH, P03/C18

The attached final report has been received from the principal investigator on the subject NIOSH grant. If this document is forwarded to the National Technical Information Service, please let us know when a document number is known so that we can inform anyone who inquires about this final report.

Any publications that are included with this report are highlighted on the list below.

Attachment
cc: Sherri Diana, EID, P03/C13

List of Publications None

Title:	In Vitro Assay for Hapten-Specific Priming of Human T Lymphocytes
Investigator:	J. Wayne Streilein, M.D.
Affiliation:	Schepens Eye Research Institute
City & State:	Boston, MA
Telephone:	(617) 912-7422
Award Number:	5 R03 OH003650-02
Start & End Date:	9/30/1998–9/29/2000
Total Project Cost:	$81,000
Program Area:	Allergic and Irritant Dermatitis
Key Words:	

Abstract:
Our results have demonstrated that T cells in lymph nodes draining skin sites painted in vivo with haptens or irritants can be stimulated to proliferate in vitro by anti-CD3 antibodies. However, when the T cells are tested at 5 days after epicutaneous application, only cells from hapten-painted donors proliferate and secrete IFN-g in response to anti-CD3. Whether hapten-sensitized or activated by an irritant, similar proliferative properties are exhibited in response to anti-CD3 stimulus and T cells can secrete IFN. These data are consistent with the hypothesis that, under the conditions of these experiments, only primed "memory" hapten-specific T cells are detectable at 5 days after hapten sensitization. Unfortunately, no similar distinction between hapten and irritant was detected when Langerhans cells, treated with hapten or irritant, were used to stimulate unprimed (naïve) T cells in vitro. Upon repeated testing, experiments attempting to use in vitro sensitization of T cells as an assay to distinguish hapten from irritant, proved to be inconsistent. We, therefore, turned our attention to the effects of irritants and haptens on antigen-presenting cells. Our results are encouraging in this direction since we detected a significant difference in the ability of APCs to produce IL-12 (a key cytokine known to be involved in the induction of CH) when exposed to a hapten as opposed to an irritant. This result was further amplified if the treated APCs were then cultured with naïve T cells. We believe that these results indicate that strategies to distinguish haptens from irritants in vitro should be directed at their differing effects on APCS, rather than examining the eventual consequence on T cells.

Publications
No publications to date.