QUANTITATION OF CONTACT SENSITIVITY RESPONSES IN THE MOUSE BY ^{3}H-THYMIDINE LABELING OF LYMPHOCYTES

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ABSTRACT

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By

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Quantitation of delayed hypersensitivity responses has usually required the measurement of crude skin reactions. In a new ear assay for delayed contact sensitivity, mice were sensitized to sheep red blood cells (SRBC) and injected with ^{3}H thyminedeoxyribose (^{3}H TdR) to label antigen sensitive cells. The accumulation of labeled cells in ear challenge sites at twenty-four hours was then assessed by scintillation counting. Positive responses were obtained when one ear was challenged with SRBC and the other with phosphate buffered saline as a control, However, a consistent response could not be demonstrated when non-crossreacting erythrocytes were used in the control challenge. Modification of the assay permitted the detection of contact sensitivity induced by dinitrochlorobenzene (DNCB) skin painting. The method was shown to be capable of detecting this cellular response by producing consistent responses. Specificity was tested in two ways. Following challenge, DNCB sensitized mice showed an increased response to DNCB when the control was provided by phenylenediamine (PD), a second contact sensitizing chemical. If the animals were sensitized to PD however, response to DNCB was minimal. When the animals were made tolerant to DNCB by a 15 mg intravenous injection of dinitrobenzene sulfonic acid (DNBS), the response was again largely erased.

Unexpectedly, subtolerogenic (1 mg) amounts of DNBS were found to enhance response to DNCB if injected intravenously during or prior to sensitization. It was concluded that the assay is antigen specific and is detecting labeled cells, probably of the recirculating lymphocyte type.

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INTRODUCTION

Cell mediated immunity and delayed hypersensitivity (DH) have been studied in many systems, but after decades the most widely used *in vivo* detection methods are still those which measure visible or gross manifestations. Because of the complexity of cell mediated reactions and the lack of production of any one definitive soluble mediator in these reactions, macroscopic tests such as measurement of induration, footpad swelling, and ear thickening, in all likelihood will be in use for years to come. Nevertheless, there exist at present a number of alternate techniques.

The subject of this study involves one such method in which uptake of the radioactively labeled nucleotide ^{3}H thyminedeoxyribose (^{3}H TdR) by proliferating lymphocytes is used as an index of deoxyribonucleic acid synthesis and has been shown to correlate to DH (62). The validity of the labeling procedure rests on two premises: 1) the labeled cells or at least some of them are specifically sensitized to antigen and 2) the labeled cells largely retain their label during the course of the experiment. Indeed, these assumptions have been borne out in several studies (7,9,68) and are the subject of an extensive review (27). Evidence exists that animals with competent, circulating, labeled lymphocytes, upon appropriate challenge, exhibit a characteristic localized accumulation of labeled cells (29,42,63). In a test designed to measure delayed responses to purified protein derivative (PPD), the observations concerning cell labeling were applied to sensitized cells in guinea pigs (62) and

recently extended to measuring the delayed response to sheep red blood cells (SRBC) in mice (73). Unfortunately, specificity was not satisfactorily tested in this latter report. Experiments reported herein, using both SRBC and a control antigen challenge in each mouse, could not substantiate claims of sensitization to SRBC. Recently, contact sensitivity to simple chemicals has emerged as one of the most widely used means of studying DH as well as T cell specificity and function in the mouse. Advantages of this system are 1) sensitivity is easily induced, 2) a nearly pure T cell response is evoked, 3) the antigenic determinant is simple and defined, and 4) a gross response measureable by conventional means is produced. Several recent reports have utilized this system to study various aspects of contact sensitivity (3,4,65,66) by measurement of ear thickness of challenged non-sensitized animals vs challenged sensitized animals as a gauge of response.

The work described in this paper represents the adaptation of the ^{3}H thymidine assay to the measurement of contact sensitivity in a procedure employing more rigorous controls than those used previously (5,65, 73) by providing both a test and control challenge in each mouse. Specificity has been tested by a reverse procedure in which mice are sensitized to the control antigen and has also been tested in animals made tolerant to the sensitizing agent. The finding that subtolerogenic levels of DNBS, used to induce tolerance, actually enhances *in vivo* responses is discussed.

REVIEW OF THE LITERATURE

Historical Perspective of Delayed Hypersensitivity

Recognition of the Phenomenon

Cellular hypersensitivity, a form of cellular immunity, may be defined as an inflammatory reaction which is immunologically specific, takes hours to reach maximum intensity and occurs in the absence of demonstrable antibody. Because they meet this definition, reactions such as homograft rejection, tumor suppression, contact hypersensitivity, experimental autoallergy, and bacterial hypersensitivity are now generally included in this category. Extensive study of the latter in the classic tuberculin reaction has led to the term delayed hypersensitivity which is often applied to this class as a whole. Various aspects of DH have been touched upon or described throughout the history of medical science. Jenner was probably the first to describe the reaction under controlled conditions. He noticed a 'reaction of immunity' in revaccinated persons which reached a peak intensity within twenty-four to seventy-two hours. In 1890, Koch provided one of the earliest descriptions of the hypersensitivity reaction produced by subcutaneous injection of tuberculin in tubercular patients. He was also the first to recognize the diagnostic value of the reaction. Although he used fever to detect reactions, the skin response became the standard in the study of tuberculosis for the next 30 years.

Zinsser (80) recognized that the tuberculin reaction represented a

principle applicable to bacterial infections in general. This work was supported by Dienes (21) who studied the histological aspects of the tuberculin reaction which he characterized as a slow exudative phenomenon with an early infiltration by mononuclear cells. He contrasted this to the anaphylactic reactions in which there was a rapidly developing edema followed by intense polymorphonuclear infiltration. He found that ordinary proteins can produce much the same effect and the best method of producing this DH was to inject the antigen directly into the tuberculous focus (20).

Another form of DH is the skin reaction resulting from contact by simple chemicals. Although cases of contact sensitivity to such diverse materials as East Indian Satin wood and mercury were reported in the early 1900's, the mechanism of this sensitivity was not systematically investigated until Landsteiner's work in the 1930's (46). Working with a number of chloro- and nitro- substituted benzene products, he found that substances containing loosely bound Cl^- and NO_2^- which would interact with amino groups of the organic base aniline were the substances that produced contact sensitivity in guinea pigs. The analogy drawn was that the simple chemicals were built up into antigens by attachment to proteins *in vivo*. Landsteiner was also able to produce contact sensitivity in guinea pigs by infecting picryl chloride conjugated erythrocyte stroma along with tubercle bacilli as adjuvant. This suggested to him that the reaction must be related to bacterial type allergies. The formal link was provided when Landsteiner and Chase (47) passively transferred contact sensitivity to picryl chloride in guinea pigs by peritoneal exudate (PE) cells as was done for tuberculin hypersensitivity (18).

Brent (15) demonstrated that homograft reactions were also related to these phenomena. Skin grafts normally tolerated by the host were

rejected if the host were first injected with lymphoid cells sensitized to donor skin. Furthermore, an inflammatory response resembling the tuberculin reaction was produced when lymphoid cells from a homograft recipient were injected into the skin of the donor.

Recognition of the Cell Type Involved in Delayed Responses

The cell eventually implicated as the antigen sensitive cell in cellular responses was the circulating small lymphocyte. Some of the earliest evidence of this was provided by experiments in which it was shown that passively transferred small lymphocytes obtained from the thoracic duct or peripheral blood of mice and rats were capable of producing a graft vs host (GVH) response or enhancing homograft rejection (10,34). Abundant evidence now exists equating these recirculating lymphocytes with thymus derived (T) cells. This point is most strikingly emphasized by the reduction or ablation of cellular reactions such as homograft rejection and GVH reaction in T cell suppressed, neonatally thymectomized, or congenitally athymic animals (34,44).

The class of cells designated as T cells is today under intense scrutiny. Various types of heterogeneity has been ascribed to these cells as a result of these studies. Raff and Cantor (67) have proposed an arbitrary designation of T_1 and T_2 cells on the basis of migration in irradiated recipients, ability to react to histoincompatible cells in a GVH reaction, and amount and type of surface antigens by reaction against specific antisera. Blomgren and Andersson (12) have found in mice a cortisone resistant fraction of thymus cells which accounts for only 3-5% of the total thymus, yet displays in GVH and antibody helper function assays a response equal to that of an intact thymus. Discontinuous density gradient separation of thymus cells by Levey and Burleson (49) has revealed

further heterogeneity. When several parameters, including ^{3}H TdR uptake in response to antigen stimulation, cell proliferation in response to mitogen stimulation, and ability to mount a GVH response were applied, only three of nine fractions were active in one or more of these properties. On another level Cantor and Asofsky (16) have presented evidence for the synergism of two types of T lymphocytes in GVH reactions in mice. In a regulatory role, suppressor T cells have been reported (31) which are able to reduce specifically both antibody response and T cell proliferation. These cells may represent another subpopulation of T cells.

Characterization of Delayed Hypersensitivity

A typical delayed reaction can be described as follows: the reaction begins at four to six hours and peaks at twenty-four to forty-eight hours. Gross manifestations are induration, erythema, and even necrosis in severe reactions (76,77). One of the earliest effects is an increased permeability of blood vessels resulting in the accumulation of fluid and cells in the tissue containing antigen. The area of infiltration is determined largely by the extent of antigen diffusion. Infiltration by mononuclear cells increases rapidly over twenty-four hours. At the peak of infection 90% of these cells are mononuclear. In a study by Najarian and Feldman (62), guinea pigs sensitized to PPD or other proteins, injected with ^{3}H TdR and subsequently skin tested, have been shown by autoradiographic techniques to contain fewer than 10% specifically sensitized lymphocytes at the site of inflammation. Kosunen et al. (43) in a similar type of experiment have reported higher values. Remaining cells were mainly macrophages which exhibit activation characterized by increased size, a higher level of pinocytosis, enzyme changes, and stickiness (77).

The aspect of DH most vigorously pursued in recent years has been

the mechanism responsible for this type of reaction. At least partial success in this regard can be claimed in the discovery of a number of soluble mediators of inflammation and tissue damage expressed in delayed reactions. In early studies, little importance was placed on making a distinction between lymphocytes and macrophages involved in DH reactions. The work by Rich and Lewis (69) 40 years ago showed that when spleen or lymph node cells from sensitized guinea pigs were put *in vitro* with antigen, cell migration was inhibited. More recently David (19), George and Vaughan (30), and Bloom and Bennet (13) showed that this inhibition is the result of macrophage inhibition factor (MIF) elaborated by lymphocytes. Since the discovery of MIF, it has been demonstrated that antigen contact with sensitized lymphocytes is the initiating event for the production of a number of other soluble factors as well. Among these are chemotactic factors (78), lymphotoxic factors (71), growth inhibitory factors (48), and mitogenic factors (48). Discovery of these mediators has strengthened the link between the various forms of cellular hypersensitivity and has served to explain various manifestations of DH, but consequently has proven to be both a blessing and a curse. Their varied effects have opened a number of investigative avenues of approach to DH but at the same time have added another dimension to its complexity.

In Vitro Measurement of Delayed Hypersensitivity

Elucidation of events associated with antibody production came about in a very short time. This was due largely to the development of the hemolytic plaque assay (40) which allows precise quantitation of antibody producing cells. Unfortunately, no comparable technique exists for measurement of DH, largely because of the lack of a mediator produced in

stoichiometric amounts and the sheer complexity of the reaction. Consequently a great deal of effort has been devoted to the study of various aspects of this reaction *in vitro*. A comprehensible review cannot attempt to cover all research conducted in this area but may point out key experiments, publications and reviews. The essential criterion in all of the following methods is a correlation to an *in vivo* characteristic of cellular hypersensitivity. In general, most *in vitro* techniques are systems which are initiated by interaction of antigen and sensitized lymphocytes and result in a change in the lymphocyte population which is measured morphologically, by the production of soluble substances, or by an effect on target cells. Particular systems vary from laboratory to laboratory and ideally attempt to measure one element of DH. These can be categorized by five general parameters.

Blast Transformation

Upon confrontation with antigen or mitogens, a certain number of exposed lymphocytes undergo a blast transformation. These cells differentiate to a lymphoblastic form characterized by the appearance of nucleoli, increased basophilia, diffuse chromatin, and eventually increased numbers of mitotic figures (50). The assessment of blast transformation is technically very simple, and is usually run by placing lymphoid cells in culture with antigen or mitogen. After seventy-two hours, transformation is evaluated by staining and observing morphological features (vide supra) or monitoring increased RNA, DNA, or protein synthesis by measuring uptake of radioactive precursors. Supernatants of such cultures may also be tested for 'blastogenic factor' by addition to cultures of normal lymphocytes and observing the effect (22,56).

Inhibition of Macrophage Migration

Early studies of tuberculin sensitivity indicated that addition of antigen to cultures of leukocytes from tuberculin sensitized animals was more cytotoxic than addition of antigen to normal cultures (38). Rich and Lewis (69) found that tuberculoprotein also caused an inhibition of migration of cells from spleen and buffy coat. George and Vaughan (3) developed the capillary tube method to make the procedure more quantitative. David (19) was able to show that this effect was due to the soluble mediator MIF, and was produced by sensitized lymphocytes eight or more hours after antigen contact.

Macrophage Activation

Activation has been described by various parameters such as increased adherence to glass, cell size, particle uptake, lysosomal enzymes, and ameboid activity. These changes have been linked to release of soluble factors by lymphocyte-antigen interaction (1,60), but the number of factors, the specificity of each, and the isolation of each are problems yet to be resolved. Increased activity in clearance of bacteria and other particulate antigens has generally been considered nonspecific (52), although an increasing number of reports have appeared attesting to specificity in antigen clearance (8) as well as cytotoxicity (25).

Interferon

Certain antigens or mitogens upon contact with sensitized lymphocytes cause the release of a factor known as interferon. This factor bestows on lymphocytes a protective effect against a number of viruses and is measured by plaque reduction or end point dilution (36).

Cytotoxicity and Inhibition

Cytotoxicity and inhibition have been the most frequently used *in vitro* methods because of technical ease and the obvious parallel of tissue damage and cell death in the *in vivo* situation. A number of individual methods exist for determining cytotoxicity including gross observation (33), enumeration of plaques in a monolayer (51), cell counts (79), and isotope release (64). These methods have been employed by many researchers in a variety of systems. Confusion exists because of the large variety of techniques in use. This introduces many variables, most of which concern the purity of the effector cell population and the type of target cells. For example, the use of spleen, lymph node, thoracic duct, or PE as a source of effector cells by different investigators affects results because these sources differ in proportion of contaminating bone marrow derived (B) cells and may well differ in T cell activity. Various systems also differ as to whether macrophages, sensitized lymphocytes, or a combination of the two effect cytotoxicity. The combination of these unanswered questions and the inherent difficulty in correlating numbers of cells killed in these different systems to an *in vivo* delayed reaction makes interpretation of results difficult.

Obviously the above methods are useful for quantitating responses within a particular system. However, by way of generalization it may be said that while *in vitro* methods exhibit a broad correlation to the *in vivo* phenomena, meaningful quantitative extrapolation to the *in vivo* situation is difficult.

In Vivo Measurement of Delayed Hypersensitivity

Cell mediated immunity is demonstrated *in vivo* by a number of different measureable reactions. These include GVH reactions, transplantation

rejection, tumor suppression, and skin reaction. All have been successfully induced in humans, guinea pigs, and rats. While the mouse is the classic animal for transplantation and tumor studies (11,57), delayed skin reactions, which have proven to be one of the most useful DH manifestations in guinea pigs, are not consistently produced (5,35). This problem has been circumvented to some extent by measuring footpad swelling or ear thickening instead of measuring an area of induration on the skin, although success has been claimed with skin reactions (32). It is quite evident that assays of this type in any experimental situation are qualitative because the measureable effects of DH are produced largely by nonspecific cells. These methods are influenced by the activity of the lymphokine-producing cells and the number of macrophages participating in the reaction. In attempts to obtain more quantitative information about delayed reactions, several researchers have made use of ^{3}H TdR uptake by proliferating cells as a measure of DNA synthesis. Tritiated thymidine uptake by lymphocytes responding to a variety of antigens has been demonstrated both *in vivo* and *in vitro* (7,23,63). Studies indicate that in a syngeneic system, labeled lymphocytes are stable and reutilization presents little problem for several days (9,27,68). Several experiments have been described in which guinea pigs were sensitized to PPD, an antigen evoking a good delayed response, and then injected with ^{3}H TdR. Lymphocytes passively transferred to normal recipients, upon ear challenge were found to produce visible skin reactions. Correlation of ^{3}H TdR uptake to DH was provided by autoradiographic techniques which demonstrated that 2-12% of the responding mononuclear cells were labeled while a maximum of only 0.8% were labeled in normal animals when challenged with the same agent (61,62). Two other groups were unable to confirm these

observations (37,41). McCluskey et al. (54) found labeled cells at specific challenge sites to average about 4% of the total mononuclear infiltrate. This group concluded that the small number of specifically labeled cells necessary to elicit a delayed response made a mechanism of specific attraction for these cells unnecessary. Najarian and Feldman (61) further explored the problem by injecting mixtures of labeled tuberculin sensitive cells and unlabeled dinitrochlorobenzene (DNCB) sensitive cells into normal guinea pigs and studying the appearance of labeled cells at reaction sites. When the test antigen corresponded to the competency of the labeled cells, a greater percent of the infiltrating cells was labeled. These data indicated that cells may accumulate at the site randomly but sensitized cells are retained more efficiently. Sprent, Miller and Mitchell (75) in an adoptive transfer system were also able to show localization of sensitized circulating lymphocytes in lymph nodes shortly after intravenous (i.v.) injection of sheep red blood cells (SRBC). Ford and Marchesi (29) have similarly demonstrated localization and the selective disappearance of antigen sensitive thoracic duct cells in mice when footpads were injected with SRBC.

Early *in vivo* studies of DH were carried out largely with tuberculin, bacteria, or contact sensitizing agents. A number of other antigens have since been employed in several systems. Asherson and Ptak (5) reported ear swelling in mice with protein antigens in addition to a number of contact sensitizing chemicals. It has been reported that low doses of some antigens stimulate T cells while B cells are left unaffected (59, 74) and that this same dose optimally produces DH in mice (42). This was demonstrated in the latter report by footpad swelling in mice sensitized with a low dose of SRBC. Sabolovic et al. (73), in mice, have

used SRBC and ^{3}H TdR labeling in an ear assay similar to that used earlier with PPD in guinea pigs (62). SRBC were injected subcutaneously (sq) and one day later injected with ^{3}H TdR intraperitoneally (ip). Ears were then challenged by intradermal (id) injection of the test antigen and the saline control. Twenty-four hours later the ear tissue was removed and prepared for scintillation counting. An increase of activity in the antigen challenged ear was shown; however, specificity of the response was not demonstrated because only saline was used as a control. In a variation of the ear assay, Asherson and Allwood (3) prepared picryl chloride sensitized lymph node cells, labeled them nonspecifically *in vitro* with ^{51}Cr and injected these cells into recipients. Assay showed that cells from immune animals accumulated at a site of challenge to a much greater degree, but this method did not distinguish antigen-sensitive cells and hence the question of specificity was not answered.

In summary, it is clear that cellular responses are varied and complex. While several forms of the classical skin reaction have been and still are widely used to measure this type of response *in vivo*, it must be recognized that results obtained are at best only qualitative. Quantitation is difficult since no one factor is produced in amounts easily measureable. Only relatively recently have investigators utilized radioactive labeling in this problem. This was done either by specifically or nonspecifically labeling lymphocytes which are capable of passively transferring DH, and then measuring accumulation of label in locally challenged tissue. Unfortunately, controls have often been neglected in this type of experiment, leaving the procedure short of proven and specificity of the results in doubt.

MATERIALS AND METHODS

Mice

(C3H/He x C57BL/10z)F_1, abbreviated BCF_1, and CBA/J female mice, two to four months old, were used for all experiments. BCF_1 mice were obtained from Health Research Inc., Seneca, New York or Cumberland View Farms, Clinton, Tenn. and CBA mice from Jackson Laboratories, Bar Harbor, Maine.

Irradiation

Mice were exposed to 1000 rads of ^{60}Co γ-radiation from an emitter contained in the Department of Food Science facility at Michigan State University. Mice were rested at least 4 hours before transplantation.

Antigens

Sheep red blood cells (SRBC), chicken red blood cells (CRBC), and goat red blood cells (GRBC) were preserved in Alsever's solution and washed three times with phosphate buffered saline (PBS) before use. 1-chloro-2,4 dinitrobenzene (DNCB) and 2,4-dinitrobenzene sulfonic acid (DNBS) were purchased from Eastman Kodak Co., Rochester, New York and the latter was recrystallized as follows. Fifteen grams of the salt was dissolved in a liter of 95% ethyl alcohol by heating to 70° C. Powdered charcoal was added and the mixture stirred and filtered through three thicknesses of Whatman No. 3 paper on a Buchner funnel. DNBS crystals appeared on slow cooling. This procedure was repeated a total of three

times. Phenylenediamine (PD) was purchased from Fisher Scientific Co., New Jersey.

Cell Suspensions and Transplantation

Nucleated spleen, bone marrow and thymus cells were suspended in Eagles medium (MEM) and counted with the aid of an eosinophil counting chamber. 6.7×10^7 cells were injected into a lateral tail vein of irradiated mice. The DNCB sensitization procedure was begun two days later.

Immunization

SRBC immunization was carried out by iv or sq injection of the antigen as indicated. 5×10^8 SRBC were considered a routine dose and 2×10^5 SRBC a low dose. Animals were injected once at the beginning of the experiment. Hapten sensitization was carried out by painting the clipped abdomen of mice with four drops of a 1.5% solution (3% in experiment reported in Table 3) of DNCB or PD in olive oil on days 1 and 3. The solution, in olive oil, was dropped onto the skin by syringe and 21 gauge needle.

Radioactive Labeling

^{3}H TdR, specific activity 20 Ci/mmole, was purchased from New England Nuclear, Boston Mass., and diluted to 10 μ Ci/ml shortly before use. Mice were given three 1.5 μ Ci injections ip at eight to ten hour intervals beginning 1 1/2 days before challenge unless otherwise indicated.

Ear Assay

Challenge with the erythrocyte antigens was done by id injection of 5 μ l of a 50% suspension in the pinna of the ear by means of a microliter

syringe and 30 gauge needle. Challenge by DNCB or PD in olive oil was accomplished by applying one drop of a 1.5% solution of the agent to the ear and spreading over both surfaces with the flat of the needle. Twenty-four hours after challenge, pinnae were excised with a scissors along the base of the ear and solubilized in 1 ml of Soluene (Packard Co., Downers Grove, Ill.). Following solubilization at room temperature, 15 ml scintillation fluid consisting of 4 g 2,5-diphenyloxazole (ppo) and 50 mg 1,4-bis[2-(4-methyl-5-phenylorazolyl)] benzene (dimethylpopop; Research Products International Corp., Elk Grove, Ill.) in one liter toluene was added. The vials were stored in the dark for one week to eliminate chemoluminescence (27) and counted in a Packard model 3320 scintillation counter.

Measurement of Cell ^{3}H TdR Incorporation

Cells were harvested from mice at the time the ear assay would normally be performed. Pooled spleen suspensions for each group were prepared in MEM and counted with the aid of an eosinophil counting chamber. PE cells were harvested by injecting donor mice ip with three ml of PBS shortly before sacrifice. The cell suspension, which was first aspirated by syringe from the abdominal cavity upon sacrifice, was passed through a glass wool column formed from a 10 ml syringe to remove adherent cells. Aliquots of 0.1 ml were solubilized in 1 ml of Soluene and prepared for scintillation counting in the same manner as ear tissue *(vide supra)*.

Assay for Plaque-forming Cells

The number of direct (19S) and indirect (7S) plaque-forming cells in spleens of DNCB sensitized mice was determined on day 6 by the Jerne hemolytic plaque method (40) as modified for use with agarose gel on

glass microscope slides. Details of the procedure are described by Miller and Cudkowicz (55).

Evaluation of Ear Responses

The following formula was used in the calculation of specific challenge responses:

$$\% \text{ INCREASE} = \frac{\text{CPM (TEST EAR)} - \text{CPM (CONTROL EAR)}}{\text{CPM (CONTROL EAR)}} \times 100$$

Negative responses were obtained when counts per minute (CPM) in the control ear exceeded CPM in the test ear. An increase of 10% or greater was designated a positive response and is based largely upon the residual response obtained from tolerant animals and animals sensitized to PD, the control antigen.

Coupling of DNBS to SRBC

The procedure followed is described by Rittenberg and Pratt (70). Briefly, PBS washed SRBC are further washed three times in modified barbital buffer (MBB) and 3 ml of the erythrocytes were added to a solution of 60 mg DNBS in 21 ml cacodylate buffer. The mixture was magnetically stirred for 10 minutes and washed three times in cold MBB containing glycyl-glycine. Although cells were stable for 3-4 days, they were washed again if not used immediately.

Preparation and Use of Antithymocyte Serum

Rabbit antithymocyte serum (ATS) was prepared by injecting two rabbits twice with 10^9 BCF_1 thymocytes iv at an interval of two weeks. Rabbits were bled following the second injection. ATS was heat inactivated by incubating for one hour at 56^o C and then adsorbed by incubating

for one hour at 4^{o} C at a volume ratio of 1:3 with BCF_1 liver previously washed three times in PBS. Mice were injected iv with 0.1 ml of undiluted serum on days 1 and 3 of sensitization.

RESULTS

Evaluation of SRBC Ear Assay

The ^{3}H TdR ear assay in CBA mice has been reported to be antigen specific (73) when animals are sensitized to SRBC. This antigen has also been used in another system whereby doses as low as 10^{5} SRBC have been reported to produce an optimal sensitization for DH when measured by footpad swelling (42). To test this ear assay as well as low antigen sensitization, SRBC in both routine and low doses were used to sensitize two groups of BCF_1 mice. A third control group received no antigen. All animals were injected with 2 μ Ci ^{3}H TdR on day 2, challenged with PBS in the left ear and SRBC in the right ear on day 4, and assayed on day 5. The results, expressed in Table 1, show a greater accumulation of counts in antigen challenged ears than in PBS injected control ears for both sensitized groups while virtually no increase was seen in the nonsensitized group.

In a second experiment, a protocol similar to that used by Sabolovic (73) was used in which a group of CBA mice was immunized by a sq injection of SRBC. Following ^{3}H TdR labeling, the animals were challenged with CRBC as the control in place of PBS. Responses, displayed in Table 2, indicate no pattern of increased label accumulation in the SRBC challenged ears occurred when a non-crossreacting RBC served as a control. Similar preliminary experiments employing either GRBC or CRBC as the control also failed to provide evidence of selective label accumulation in SRBC challenged ears and hence claims of specificity within this system remain unsubstantiated.

Table 1. Comparison of ear assay responses for routine and low dose immunized mice challenged with PBS and SRBC

Group	Treatment+	CPM@/PBS ear	CPM/SRBC ear	% Increase	Fraction positive (>10%) & Mean ± SE
A	SRBC* (routine dose)	4706	5072	7.7	3/5
		5073	5431	6.2	
		4409	4882	10.6	
		3570	5601	57.0	
		4389	5278	19.8	20.2 ± 9.5
B	SRBC* (low dose)	4111	4327	5.2	3/4
		3686	6487	76.5	
		3316	3986	20.5	
		3760	5051	34.3	33.9 ± 15.4
C	No antigen	2566	2333	-8.2	0/4
		3430	3418	0.0	
		3995	4373	9.5	
		4234	4352	2.8	1.0 ± 3.7

+ CBA mice; 2 μCi ^{3}H TdR injected ip day 5

@ Counts per minute

* Injected iv; assayed day 5

Table 2. Ear assay responses of mice* immunized sq with a routine dose of SRBC and challenged with CRBC and SRBC

Mouse number	CPM/CRBC ear	CPM/SRBC ear	% Increase	Fraction positive (>10%) & Mean ± SE
1	1175	981	-16.5	1/10
2	1805	1528	-15.3	
3	1324	1120	-15.4	
4	1729	1495	-13.5	
5	1410	1798	27.4	
6	1069	1119	4.6	
7	1673	1580	-5.5	
8	1569	1456	-7.2	
9	1476	1579	7.0	
10	1068	895	-16.1	-6.5 ± 4.5

* CBA mice injected with 5 x 10^8 SRBC; 1.5 μCi ^{3}H TdR injected ip day 2; assayed day 5

Measurement of Delayed Contact Sensitivity by Ear Assay

Upon analysis of the previous experiments, failure to demonstrate specificity was attributed to one or more of a number of possibilities: 1) an untenable assay, 2) inability of the SRBC antigen to induce adequate DH, 3) interference by the trauma of ear challenge whereby tissue damage results in nonspecific accumulation of labeled cells, and 4) leakage from the challenge site resulting in unequal antigen distribution from mouse to mouse. It was reasoned that another type of delayed response, contact sensitivity to DNCB, would provide a more objective evaluation of

the assay by eliminating the last three possibilities since DNCB is known to induce a delayed response and can be applied topically. With reference to Table 3, groups A and B were sensitized with low or routine doses of SRBC as before while a third group was skin painted with DNCB. Animals received two injections of ^{3}H TdR in an eight hour period. Groups A and B were challenged with CRBC and SRBC by id injection and group C was challenged by painting the ears with either PD or DNCB. PD was chosen as the control in this system because of its simple chemical nature and its ability to produce an inflammation of the same magnitude as DNCB (5). One and four negative responses were obtained for the low and routine dose SRBC sensitized groups respectively. DNCB sensitization however, resulted in all positive responses. Furthermore the mean response was 149%, indicating that the system was capable of providing more dramatic and consistent responses than the SRBC system and warranted further study. This consistency was indeed demonstrated in the next experiment depicted by group A of Table 4. CBA mice were sensitized with DNCB, injected three times with ^{3}H TdR, challenged with DNCB and PD and assayed on day ten. Eight of nine mice responded positively within a narrow range.

Intensity and Specificity of Response to DNCB

Many studies have been carried out using skin reactive haptens to produce contact hypersensitivity. Two recent reports have emerged indicating tolerance to DNCB and dinitrofluorobenzene has been induced by iv injection of the sulfonic acid form of these chemicals, DNBS (5,65). In the system being described, specificity would be demonstrated if normal response upon challenge could be abrogated by tolerance induction. Mice in group A of Table 4 were tested by the standard procedure while mice in groups B-D, in addition to DNCB sensitization, were injected with DNBS in

Table 3. Comparison of Responses in SRBC and DNCB immunized mice challenged by a control and a test antigen

Group	Treatment	Mouse number	% Increase	Fraction positive (>10%) & Mean ± SE
A*	SRBC (low dose)	1	94	4/5
		2	-54	
		3	146	
		4	16	
		5	59	52 ± 34
B*	SRBC (routine dose)	1	-49	1/5
		2	-37	
		3	-32	
		4	-52	
		5	76	-18 ± 24
C+	DNCB	1	250	5/5
		2	214	
		3	155	
		4	24	
		5	103	149 ± 40

* BCF_1 mice injected iv; challenged with CRBC and SRBC; assayed on day 5

\+ BCF_1 mice skin painted by 3% DNCB in olive oil on days 1 and 3; challenged with PD and DNCB; assayed on day 7

Table 4. Ear responses obtained from DNCB sensitized mice: effect of varying iv DNBS injections

Group	Treatment - Day		Mouse number	% Increase	Fraction positive (>10%) & Mean ± SE
A*	DNCB	1,3	1	35.2	8/9
	&		2	-25.2	
	Assay	10	3	34.8	
			4	30.5	
			5	42.9	
			6	41.9	
			7	31.3	
			8	10.2	
			9	24.0	25.0 ± 7.0
B*	DNCB	1,3	1	15.9	8/9
	&		2	177.7	
	DNBS (1 mg)	6,8	3	32.4	
	&		4	15.3	
			5	3.9	
	Assay	10	6	63.4	
			7	110.0	
			8	130.6	
			9	245.3	88.3 ± 27.7

Table 4. (cont'd)

Group	Treatment - Day		Mouse number	% Increase	Fraction positive (>10%) & Mean ± SE
C*	DNCB		1	78.2	10/10
	&		2	72.8	
	DNBS (1 mg)	-6,-5, -3,1,3	3	23.7	
	&		4	83.0	
	Assay	9	5	74.7	
			6	52.1	
			7	75.8	
			8	113.7	
			9	137.0	
			10	80.5	79.1 ± 9.7
D+	DNCB	1,3	1	6.8	3/8
	&		2	-1.9	
	DNBS (15 mg)	7	3	0.1	
			4	-2.1	
			5	10.5	
			6	-0.4	
			7	31.6	
			8	11.4	7.0 ± 4.0

* CBA mice

+ BCF_1 mice; assayed on day 7 in protocol similar to that in earlier experiment employing BCF_1 mice (Group C, Table 3)

a progressively more rigorous regimen for the purpose of inducing tolerance. Group B was injected with 1 mg iv twice during the DNCB sensitization period and group C three times before and twice during sensitization. The data demonstrate no suppression occurred and in fact, enhancement was observed. Mean responses were increased at least two-fold for each group and with a remarkable consistency as attested to by the fact that there were no negative responses in nineteen animals tested. Eighteen of these exhibited responses greater than 10%. Group D mice were injected with 15 mg of DNBS 7 days before DNCB sensitization. Due to the unavailability of CBA mice, BCF_1 mice were used in this group and assayed on day 7 instead of day 10 because this had been the protocol in an earlier experiment involving BCF_1 mice (Table 3, Group C). Only one response was well above the 10% level and the mean was 6.9% for the group, indicating that a state of specific unresponsiveness had been induced and had resulted in the abrogation of ear response to DNCB.

In a subsequent experiment shown in Table 5, group A was sensitized with PD instead of DNCB. Mice were challenged on day 8 with PD and DNCB as before. Although response to DNCB was not completely eliminated, individual response and the mean were considerably reduced and are at the same level as the residual response found in tolerant animals. This small positive response may reflect cross reactivity at the T cell level whereby PD sensitive cells are able to recognize DNCB as well as PD, but not vice versa. An alternative explanation may be that this response was merely the result of lower immunogenicity for a comparable amount of the chemical, hence a poor stimulation. Group B consisted of nonsensitized normal mice challenged with PD and DNCB. The responses are more varied than those of sensitized animals and the mean of -13.6% favors PD

Table 5. DNCB response obtained in A) PD sensitized mice, B) non-sensitized mice and, C) DNCB sensitized mice challenged with DNCB and olive oil

Group	Treatment	Mouse number	CPM/left ear	CPM/right ear	% Increase	Fraction positive (>10%) & Mean ± SE
A*	PD (day 1,3) & Assay (day 9)	1	1550	1583	2.1	6/12
		2	2021	2659	24.0	
		3	1803	1862	3.2	
		4	2068	2412	14.4	
		5	1912	2427	21.1	
		6	2467	2450	-0.7	
		7	2087	2007	-4.0	
		8	1933	2285	-15.4	
		9	2001	2290	12.6	
		10	2092	2112	0.9	
		11	1997	2287	12.7	
		12	1875	2156	8 8	9.2 ± 2.5
B+	No sensitization & Assay (day 9)	1	2348	2441	4.0	1/5
		2	2695	1298	-51.8	
		3	2314	1475	-36.3	
		4	2070	2665	28.7	
		5	2319	2027	-12.6	-13.6 ± 13.4

Table 5. (cont'd)

Group	Treatment	Mouse number	CPM/left ear	CPM/right ear	% Increase	Fraction positive (>10%) & Mean ± SE
C*	DNCB (day 1,3) & Assay (day 9)	1	2856	4017	40.7	4/4
		2	2010	2988	48.7	
		3	2069	4160	101.1	
		4	2380	4017	58.5	62.2 ± 13.4

* CBA mice; challenged with PD (left ear) and DNCB (right ear)

+ CBA mice; challenged with olive oil (left ear) and DNCB (right ear)

challenge. Group C mice were sensitized with DNCB and challenged with olive oil, the suspending medium used for sensitization and challenge, in addition to DNCB. This mean compared to the mean response obtained in the routine procedure (Table 4, Group A) is within the same range, indicating that PD cross reactivity does not significantly affect DNCB response.

Determination of the Mediator Cell Source

To determine which type of cell could account for the accumulation of ^{3}H labeled cells in ear responses, the following experiment was performed. Spleen, bone marrow, and thymus cells were collected from normal BCF_1 mice and 6.7×10^{7} of each type were injected into irradiated recipients. These mice were then sensitized to DNCB by skin painting and on day 7, spleens of each group were collected and cell suspensions prepared and pooled. Results showed that 3.5×10^{-3}, 2.4×10^{-3}, and 4.5×10^{-3} CPM/cell were incorporated by spleen, bone marrow, and thymus cells

respectively. Although these results indicated slightly more label was taken up by thymus cells, analysis showed that a moderate CPM increase of test ear over the control ear required an unreasonably large number of cells at this level of incorporation.

In a subsequent experiment, normal mice were sensitized to DNCB and on day 6 both spleen and PE cells were collected and pooled separately. Peritoneal cells were further treated by filtering through a glass wool column to remove macrophages. Aliquots of both spleen and peritoneal cells were then optically counted and prepared for scintillation counting. Peritoneal cells were found to yield 2.2×10^{-2} CPM/cell while the value for spleen cells was 4.2×10^{-4} CPM/cell. This suggested that peritoneal lymphocytes, which consist of the circulating type T cell as opposed to the non-circulating type found in the spleen, are a likely source of the reactive cells in the ear response. Furthermore, a reasonable number of these cells contain enough label to account for the increases of ^{3}H observed in ear responses.

Inhibition provides another means of determining the source of cells mediating this sensitivity. An agent at least partially selective in suppressing T cells should inhibit the response to DNCB if this is a T cell phenomenon. Azathioprine (AZT), a 6-mercaptopurine analog, is one such drug which has been shown to inhibit certain types of DH responses (6). Table 6 indicates the responses obtained when mice received none, one, or two sq injections of AZT at the time of DNCB sensitization. Responses were considerably reduced in the mice receiving the drug treatment. However, the dosage used, 0.15 mg/g body weight, did cause several deaths and weight loss in the survivors.

In another experiment mice were treated with ATS in an effort to

Table 6. Effect of azathioprine on ear response in DNCB sensitized mice*

Treatment	Mouse number	% Increase	Mean
No AZT	1	114.2	128.7
	2	143.5	
1 AZT injection+	1	45.5	42.3
	2	33.7	
	3	47.9	
2 AZT injections+	1	61.4	69.2
	2	77.0	

* CBA mice skin painted with DNCB on days 1 and 3

\+ 2.2 mg of a 2 mg/ml solution ip/injection

show that reduction of T cells results in a coincident reduction of response since recirculating cells have been shown to be sensitive to ATS *in vivo* (44). Eight mice were sensitized to DNCB by skin painting on days 1 and 3 and were subsequently injected iv with 0.1 ml of ATS on days 6 and 8. As seen in Table 7, with the exception of animals 1 and 2, which may actually have been stimulated, responses were not significantly affected when compared to the control group with which this experiment was concurrently run (Table 4, Group A). Lack of effect by the treatment may have been due to an insufficient amount of ATS and/or injection of the antiserum at an ineffective point in the sensitization procedure. The optimal time of injection may be prior to antigen contact (16).

Table 7. Effect of *in vivo* administered ATS on response of DNCB sensitized CBA mice*

Mouse number	ATS+	CPM/PD ear	CPM/DNCB ear	% Increase	Fraction positive (>10%)
1	-	3142	4249	35.2	8/9
2	-	2143	1660	-25.2	
3	-	2743	3697	34.8	
4	-	3354	4377	30.5	
5	-	2486	3552	42.9	
6	-	2342	3314	41.9	
7	-	2205	2896	31.3	
8	-	2893	3183	10.2	
9	-	3160	3919	24.0	25.0 ± 7.0
1	+	.745	2817	278.1	8/8
2	+	1603	3159	97.1	
3	+	3170	4110	29.7	
4	+	2111	2553	20.9	
5	+	2410	3430	42.3	
6	+	2465	3489	41.5	
7	+	2660	3277	23.2	
8	+	1971	2390	21.3	69.2 ± 31.1

* This experiment run concurrently with experiment reported in Table 4, Group A and assay protocol is identical; mice 1-9 served as controls in both experiments

+ 0.1 ml ATS administered iv on days 1 and 3

Table 8. Day 6 plaque-forming cells/spleen of DNCB skin painted[@] BCF_1 mice

	Indicator cell	
	DNP-SRBC	SRBC
Direct PFC[*]	87	90
Indirect PFC	15	22

[*]Plaque-forming cell

[@]DNCB sensitization on days 1 and 3

Although not demonstrating suppression, this experiment does reinforce the response consistency obtained by this procedure.

Detection of Antibody Producing Cells

To explore the possibility that skin sensitization resulted in subsequent induction of antibody-producing cells and perhaps influenced the experimental results, a group of four mice was sensitized by DNCB skin painting. Spleens were collected on day 6 and assayed for hemolytic plaques using DNP-SRBC as a specific indicator and SRBC alone as a background control. Table 9 depicts the results. Substraction of SRBC background plaques from DNP-SRBC plaques indicates DNP-specific antibody production is not stimulated.

DISCUSSION

The objective of this study was to assess DH in mice by means of a quantitative assay. The T cells responsible for DH have two known major functions; that of collaborating with B cells through the induction of antibody responses following antigen stimulation as well as mediation of cellular immune responses. The first of these functions can be quantitatively and qualitatively assessed by ability to induce B cells into antibody production as detectable by the hemolytic plaque assay. There is yet no adequate method of quantitating the second function despite the numerous manifestations of cellular responses. A good test for DH should provide quantitative, non-subjective results. Current *in vivo* methods used in the mouse include measuring either footpad thickening, ear swelling or the degree of skin reaction--none of which yield information on cell numbers or mechanisms involved in a particular reaction.

The first assay studied was a novel technique described by Bloom et al. (14) for enumerating antigen sensitive lymphocytes obtained from tuberculin sensitized guinea pigs. The approach was designed to detect intrinsic changes in these antigen activated lymphocytes rather than to measure products or gross macroscopic changes and was based on the following observations. While it was well known that resting lymphocytes were refractory to a number of RNA viruses, phytohemagglutinin activated cells were not and permitted replication of the virus. It was reported that the same principle applied to tuberculin sensitized guinea pig lymphocytes which were placed in culture both with or without antigen for a period of

up to four days. Virus was allowed to adsorb, excess virus neutralized by antiserum, and cells plated in agar over a monolayer of virus-susceptible target cells.

Adaptation of this procedure to murine cells was the first consideration in preliminary experiments. Using bovine gamma globulin (BGG) as antigen, spleen, lymph node, and PE cells were prepared by culturing normal and sensitized cells of each with or without antigen prior to assay. Although a high background existed, generally higher responses were obtained with sensitized cells. Spleen cells produced the highest responses and were found to be the only practical source of cells due to the large number of cells required. Later it was found that background could be reduced by use of virus specific rabbit antiserum for excess virus neutralization during the procedure. During the course of extensive subsequent testing in many experiments, a number of variations were attempted with this assay because of a high incidence of technical failures and inconsistent results. These modifications included: 1) testing a number of antigens including BGG, heat aggregated BGG, human gamma globulin, BGG-SRBC, BGG-trinitrophenol, and SRBC in both routine and low doses, 2) elimination of the *in vitro* culture step to improve cell viability, and 3) preparation and comparison of various controlled cell types in lethally irradiated mice i.e., thymus, spleen, bone marrow.

For the following reasons, consideration of an alternate procedure was necessary. An assay suited to the objective of this study with regard to the investigation of T cell functions would necessarily be technically simple enough to allow testing a number of individual animals in addition to yielding results which are consistent and reproducible. This procedure clearly did not satisfy these requirements. Response and background

varied considerably from experiment to experiment and readable results were obtained in only approximately one-third of the experiments. Viable cells remaining after *in vitro* culturing may not reflect the true *in vivo* population of cells. In addition the time consuming nature of the assay and the requirement for an initially large number of cells made the testing of other than pooled sources of cells impractical.

The assay described by Sabolovic (73) and the subject of this paper is the second method evaluated. Use of SRBC as antigen posed several immediate problems. Ear challenge requires id injection of SRBC, a very delicate operation. It was found that some tissue damage, puncture of tiny blood vessels, and leakage of antigen from the injection site is unavoidable, thereby introducing a number of sources of variation.

A response could be demonstrated for SRBC in primed animals when PBS was used as a control challenge but not when non-crossreacting erythrocytes were used in place of PBS as the control challenge. This suggested that response measured in this procedure may not be antigen specific. The use of low doses of SRBC described as producing optimal DH without antibody production (26,42) was also inconclusive with regard to demonstration of specificity.

To avoid pitfalls tied to the use of SRBC, the study was pursued with DNCB, a contact sensitizing agent known to elicit a high level of a persistent sensitivity as a result of skin painting (5,24,47). Quantitation of contact sensitivity response to challenge is conventionally measured by gauging ear thickness with a micrometer. This assay has an intrinsic shortcoming as do other assays: namely, it measures a largely non-specific effect presumable initiated by specific antigen sensitive cells. Asherson and Ptak (5) by this method have shown that mice

sensitized either to picryl chloride or oxazolone respond favorably to challenge by these haptens with non-immunized animals as controls. Phanuphak et al., using the same procedure attempted to demonstrate specificity in another manner. Mice injected iv with a large amount of DNBS became tolerant to dinitrofluorobenzene (DNFB) but not oxazolone. However, mice sensitized to either DNFB or oxazolone were not tested with an agent other than the sensitizing agent.

An important modification in the current study was the addition of a specificity control for each individual animal. One ear was challenged with DNCB and the other with PD. Specificity was apparent for 1) DNCB sensitized, 2) PD sensitized, and 3) DNCB tolerant animals. The fact that a small residual response remains in tolerant or cross sensitized animals may reflect a less than complete tolerance to DNCB or less than optimum sensitization by PD in the respective experiments i.e., PD may be less inflammatory than an equal concentration of DNCB. The alternative possibility is that some degree of non-specific inflammatory potential is associated with DNCB.

Results of the experiment comparing label incorporation indicated that peritoneal cells contained fifty times as much activity as spleen cells from the same animal. Since peritoneal lymphocytes consist primarily of recirculating T cells (34), the implication is that these are the reactive cells in this response. In further studies, by the use of autoradiographic techniques, the percent of labeled cells from this source can be determined and correlated to CPM for a known number of cells. When used in conjunction with a passive transfer system to reduce background, numbers of participating cells could be determined.

The procedure described in this report, due to its dependence on

the uptake of radioactive precursor, measures a cellular response. A number of studies have implicated the recirculating T cell as the likely mediator of this response (34,76). If T cells indeed are responsible for initiating this reaction, the results of this study may lend support to the case for hapten specificity at the T cell level. The existence of hapten specific T cells at the present is one of the nebulous areas of cellular immunology. Janeway (39) pointed out that much of the evidence against hapten specific T cells is an inability to demonstrate function or a coincident involvement of antibody in a particular reaction. This can be traced back to early studies in which contact sensitivity could be induced successfully by id injection or skin painting but not by iv injection of DNP-conjugates. Mitchison (57) has described a system in which anti-lymphocyte serum treatment of protein primed donor mice was found to depress adoptive secondary antibody response to that particular protein. Response could be restored by adding hapten primed cells to the adoptively transferred cells and then boosting the recipients with the hapten-protein conjugate. It was later shown that this effect can be accounted for by specific antibody (39). Nevertheless, evidence for hapten specificity of T cells is now mounting. A study using azobenzene-p-arsonate has demonstrated hapten specific helper function and cellular immunity (2). It has also been shown that DNP-KLH primed spleen cells from mice were capable of binding DNP-BSA coated nylon fibers and is subject to inhibition by DNP-lysine or anti-theta serum (72). Phanuphak et al. (65,66) reported that ^{3}H TdR uptake *in vitro* by DNP-BSA primed lymph node cells was DNP specific and subject to specific tolerance. Furthermore, DNBS could induce lymphocyte proliferation *in vitro* in the absence of serum protein binding. While this does not eliminate the possibility

that DNBS is binding non-specific cells or cell products, it does point out the possibility that T cells may be capable of recognizing independent haptenic determinants.

In the current *in vivo* study, small doses of DNBS stimulated cellular responses while large amounts produced tolerance. The mechanism by which these chemicals affect the lymphocyte response is unknown but probably related to the fact that as a class they are very reactive and form protein conjugates readily (24). However, skin and serum proteins differ and this study shows iv injection of DNBS strengthens measured response to DNCB skin challenge. While the presence of cross-reactive DNP-protein conjugates in skin and serum cannot be ignored, it is possible that the carrier protein may play no part in specificity. The inability of a number of investigators to produce contact sensitivity by DNP-protein conjugates may then be explained by failure to provide a strong enough stimulus or the right kind of stimulus. In conclusion, the data provided by this and other studies cited, in addition to studies concerning T cell receptors, serve to point out that at present our knowledge of T cells at best is incomplete and warrants further research.

SUMMARY

A delayed hypersensitivity assay relying on the detection of accumulated radioactively labeled lymphocytes in a tissue challenge site was tested. Mice were sensitized to low or routine doses of SRBC, injected with ^{3}H TdR to label antigen sensitive cells, and then challenged in the pinna of the ear. Each mouse was challenged on one ear with SRBC and on the other with PBS. Upon ear tissue solubilization and scintillation counting, increased accumulation of the ^{3}H label was observed. However, this accumulation could not be demonstrated if the control ear was challenged with a non-crossreacting erythrocyte. The assay was subsequently modified to test contact sensitivity to DNCB, induced by skin painting. This system eliminated several possible sources of variation to provide a more objective test of this assay. Positive responses were consistently obtained by challenge consisting of DNCB, and PD as the control antigen. Specificity was demonstrated by showing that induction of tolerance to DNCB by DNBS injection largely abrogated response to DNCB challenge. Sensitization to PD and subsequent challenge by DNCB and PD, while not producing a response to PD, did largely eliminate response to DNCB. It was also shown that sensitization results in an increased uptake of ^{3}H TdR and that the label is found in cellular DNA.

APPENDIX

APPENDIX

^{3}H TdR Incorporation by Normal and Sensitized Mice

To properly assess the activity measured in the ear assay it was necessary to compare ^{3}H TdR uptake by normal and sensitized mice to determine whether more label was actually incorporated by lymphocytes of antigen stimulated mice than by lymphocytes of non-stimulated mice. One group of three BCF_1 mice was painted with DNCB. Beginning on day 5, this group and a second normal group was injected three times with 1 μ Ci ^{3}H TdR at an 8-10 hr interval. PE or spleen cells were collected in phosphate buffered saline on day 7. Half of the PE cell suspension from both groups was filtered through glass wool to remove macrophages. All cells were subsequently washed with PBS and counted with the aid of an eosinophil counting chamber. Duplicate 0.1 ml aliquots of each sample were centrifuged, the supernatants discarded, and the cells lysed by the addition of 1 drop 1 M NaOH. Volume was brought to 1 ml with saline and 0.2 ml of 50% trichloroacetic acid (TCA) added. This mixture was incubated 10 min at 4° C, the insoluble material collected on glass fiber filters, the filters dried and scintillation counting carried out in 10 ml toluene and omnifluor. Two additional aliquots of the DNCB stimulated PE sample were lysed with distilled water, incubated with 10 μg DNase for 30 min at 37° C and insoluble material collected on glass fiber filters for scintillation counting.

Results showed DNCB treatment increased ^{3}H TdR incorporation in both spleen and PE by approximately 50%. PE cells however, contained 20

times as much label as spleen. Glass wool filtering of both PE samples resulted in an 85% loss of cells, which indicated a large number of lymphocytes were lost in addition to macrophages. Consequently no conclusion can be drawn from this result. DNase treatment of a DNCB stimulated PE aliquot produced a 75% reduction in TCA precipitated counts indicating that at least the major portion of the label had indeed been incorporated into cellular DNA. Remaining counts could be attributed to incomplete lysis by the procedure used and a resultant inaccessibility of DNA to DNase.

As a whole these data imply that 1) the peripheral recirculating type lymphocyte is stimulated to a greater degree than the splenic type cell by hapten skin painting, 2) normal cells also incorporate label, but to a lesser degree, and 3) ^{3}H TdR is incorporated into DNA by these cells as expected.

Table 9. Comparison of spleen and PE ^{3}H TdR label incorporation in normal and DNCB sensitized mice

Cells	Cell Number	CPM	CPM/Cell
PE^{DNCB*}	7.2×10^{5}	839	11.60×10^{-4}
$PE^{NORM^{o}}$	5.3×10^{5}	390	7.37×10^{-4}
$GW^{+}\text{-}PE^{DNCB}$	9.1×10^{4}	41	4.55×10^{-4}
$GW\text{-}PE^{NORM}$	7.4×10^{4}	22	2.45×10^{-4}
SPL^{DNCB}	1.1×10^{6}	52	4.86×10^{-5}
SPL^{NORM}	1.0×10^{6}	31	3.12×10^{-5}
$DNase^{@}\text{-}PE^{DNCB}$	7.2×10^{5}	207	2.8×10^{-4}

* BCF_1 donors; sensitized days 1,3; 1 μ Ci TdR 3 times days 5,6; cells collected day 7

o BCF_1 donors; 1 μ Ci TdR 3 times days 5,6; cells collected day 7

\+ adsorbed on glass wool

@ PE^{DNCB} aliquot treated with DNase

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