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PRINCIPAL INVESTIGATOR: Arthur M. Dannenberg, Jr., M.D., Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
School of Hygiene & Public Health
615 North Wolfe Street
Baltimore, Maryland 21205-2179

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Cytokines are autocrine and paracrine protein hormones produced by cells in response to specific and nonspecific stimuli. They play a major role in both acute and chronic inflammatory processes, including those produced by sulfur mustard (SM). Understanding of the role of cytokines in SM lesions should lead to better therapy because various cytokine activators and inhibitors are becoming available.

In situ hybridization of the mRNA of various cytokines with radiolabeled antisense RNA probes enables us to visualize under the microscope which cells in tissue sections of SM lesions are producing which type of cytokine. This technique, therefore, demonstrates cell function histologically, even though the cells are no longer alive at the time of analysis.

During the past two years, we successfully demonstrated the mRNAs of three major cytokines in developing and healing rabbit SM lesions: Interleukin 1 (beta) (IL-1 (beta)), neutrophil attractant/activation protein-1 (NAP-1 or IL-8), and monocyte chemoattractant

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18. SUBJECT TERMS (CONT'D)

Cytokines:

IL-1	-	Interleukin 1
IL-8	-	Interleukin 8 (same as NAP-1)
IRAP	-	Interleukin 1 Receptor Antagonist (same as IL-1ra)
MCP-1	-	Monocyte Chemoattractant (Activating) Protein-1
NAP-1	-	Neutrophil Attractant/Activation Protein-1
PF4	-	Platelet Factor 4 (a member of the NAP-1 [IL-8] gene family)
RANTES	-	a member of the MCP-1 gene family
TGF (beta)	-	Transforming Growth Factor (beta)
TNF (alpha)	-	Tumor Necrosis Factor (alpha)

19. ABSTRACT (cont'd)

(activating) protein 1 (MCP-1). The macrophages and activated fibroblasts in the lesions contained the mRNA of all three cytokines, with the highest amounts in the peak lesions and decreased amounts during healing. Granulocytes contained the mRNA of IL-1 (beta) and NAP-1.

In SM lesions (but not in normal skin), epithelial cells of the surface and/or of the hair follicles contained the mRNA of NAP-1 and MCP-1. Evidently, SM induces such epithelial cells to produce the mRNA of these two cytokines, which, in turn, chemoattract and activate polymorphonuclear and mononuclear phagocytes and also activate the local fibroblasts. These three cell types, then, produce more cytokines, thereby perpetuating the inflammatory process.

We have developed a histochemical test for the production of H_2O_2 in tissue sections of SM lesions. Intact granulocytes, as well as those recently dead in vivo, were major producers of H_2O_2. Cells in the macrophage-fibroblast group also produced it in lesser amounts. No tissue destruction was seen adjacent to the cells producing H_2O_2, apparently because antioxidants in the tissues and in the extravasated serum prevented tissue damage by the H_2O_2.

We also performed experiments in which interleukin 1 receptor antagonist protein (IRAP or IL-1ra) and two leukotriene B_4 inhibitors were each injected into SM lesions. The IRAP had no detectable effects, grossly or histologically. The leukotriene inhibitors slightly reduced the inflammatory process, but did not accelerate the healing of the lesions.

During the next two years, we shall confirm and complete the studies just described and evaluate in SM lesions the mRNAs of tumor necrosis factor (TNF), GRO (a chemoattracting/activating growth factor related to NAP-1 and MCP-1), transforming growth factor (TGF) (beta) (a cytokine regulator), and other cytokines. We shall also evaluate, by immunocytochemical techniques, various cytokine proteins in tissue sections of SM lesions, in order to assess the product as well as its RNA message. Finally, these techniques will be applied to full-thickness human skin explants, exposed in vitro to SM and cultured for 20 hr, in order to identify additional cytokines that initiate this inflammatory process.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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AMD In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

April 29, 1992
Date

Arthur M. Dannenberg
Arthur M. Dannenberg, Jr., M.D., Ph.D. - P.I.

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INTRODUCTION

The mission of our Contract is two-fold: (a) To determine the role of various cytokines and reactive oxygen intermediates (ROIs) in the inflammatory process initiated by the topical application of sulfur mustard (SM), and (b) to reduce with cytokine and ROI inhibitors the damage produced by SM. This histopathological study should provide basic understanding of how and when these inflammatory mediators act in lesions produced by SM, so that the inhibitors can be applied in the right place and at the right time.

Abbreviations

SM	-	Sulfur mustard

CSF	-	Colony stimulating factor
EGF	-	Epidermal Growth Factor
GM-CSF	-	Granulocyte-Macrophage-Colony Stimulating Factor
GRO	-	a chemoattracting/activating growth factor related to NAP-1 and MCP-1 (originally MGSA: melanoma growth stimulatory activity)
IFN (gamma)	-	Interferon-gamma
IL- 1	-	Interleukin 1
IL-8	-	Interleukin 8 (same as NAP-1)
MCP-1	-	Monocyte Chemoattractant (Activating) Protein-1
NAP-1	-	Neutrophil Attractant/Activation Protein-1
TGF (beta)	-	Transforming Growth Factor (beta)
PF4	-	Platelet Factor 4 (a member of the NAP-1 [IL-8] gene family)
RANTES	-	a member of the MCP-1 gene family
TNF (alpha)	-	Tumor Necrosis Factor (alpha)

IL-1ra	-	Interleukin 1 Receptor Antagonist (same as IRAP)
IRAP	-	Interleukin 1 Receptor Antagonist Protein
L-656,224	-	Merck-Frosst-Canada's new leukotriene biosynthesis inhibitors
MK-886	-	

ROIs	-	Reactive Oxygen Intermediates
UV	-	Ultraviolet irradiation
DAB	-	Diaminobenzidine
BCG	-	Bacille Calmette Guerin
PLA_2	-	Phospholipase A_2
T3, T7 and SP6	-	DNA-dependent RNA polymerases
Sph I, Sst I, Hind III, Pst I	-	Restriction enzymes
EDTA	-	Ethylenediamine tetraacetate

EXPERIMENTAL METHODS

Preparation of unfixed and fixed frozen tissue sections

Rabbit dermal mustard lesions of various ages (see Table 1) were bisected. Lesions for unfixed frozen sections were placed into molds containing OCT compound, frozen in liquid nitrogen, wrapped in Parafilm (Amer. National Can Co., Greenwich, CT 06836) and stored at -80°C. Lesions for fixed frozen sections were placed for 4 hours in cold (4°C) 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2), washed overnight in 20% sucrose in phosphate-buffered saline (0.01 M sodium phosphate and 0.15 M NaCl, pH 7.2), placed into molds containing OCT compound, frozen in liquid nitrogen, wrapped in Parafilm, and stored at -80°C. Both the fixed and unfixed specimens were cut in a cryostat at 6 ±2 um.

For in situ hybridization, frozen sections of each type were dried with a cool hair dryer immediately after being placed on Silane-coated (Fisherbrand Superfrost/Plus) microscope slides (Fisher Scientific, Pittsburgh, PA 15219). The tissue sections were then fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 10 to 20 min at 23°C, rinsed, at 23°C, with 3X SSC, 1X SSC and 1X SSC for 5 min, each dehydrated in ascending concentrations of ethanol, dried with the hair dryer, and stored at -80°C in a slide box containing desiccant and sealed with tape. Slides stored under these conditions can be used for in situ hybridization for several months without significant loss of hybridizing mRNA. [1X SSC is 0.15 M NaCl and 0.015 M sodium citrate in DEPC water at pH 7.0 (1). DEPC (treated) water is distilled (or deionized) water, treated with 0.1% diethylpyrocarbonate at 23°C for at least 12 hr, and autoclaved for 15 min. This procedure inactivates traces of RNase (1).]

For hydrogen-peroxide histochemistry, frozen sections from cold-fixed and unfixed SM lesions were dried on slides, as described above, and used immediately or stored overnight in a sealed slide box with desiccant at -80°C.

In all of the procedures herein described, fixed frozen sections produced better results than did unfixed frozen sections.

PART A: CYTOKINES

Overview of in situ hybridization in tissue sections

The technology of molecular biology (outlined below) enables the pathologist to "see" cell functions in "dead" tissue sections by means of radiolabeled complementary probes for the cell messenger RNA (mRNA) of various cytokines.

These probes are complementary DNA (cDNA) or antisense RNA sequences that hybridize specifically with the mRNA of a given cytokine. In general, the large probes (containing 500 to 1000 nucleotides) hybridize with only 10% to 30% of the entire mRNA of the cytokine (which usually contains several thousand nucleotides). Thus, many different probes for the same cytokine can be made, any one of which would identify the mRNA of the cytokine. [Cytokines are small proteins. Identification of 10% or more of the molecule is sufficient to identify the whole.] Oligoprobes containing only 20 to 50 nucleotides are available for the in situ hybridization of many of the cytokines. However, with relatively few exceptions, it is the larger probes that have been used successfully to identify the mRNA of cytokines in tissue sections. [Sense RNA probes serve as specific non-hybridizing controls for antisense RNA probes.]

Molecular Biological Techniques

The preparation of human ^{35}S-labeled Epidermal Growth Factor antisense (and sense) RNA probes is outlined below as an example of how such probes are made.

The lyophilized plasmid, pmEGF-26F12 (ATCC 37486), was received from the American Type Culture Collection (ATCC) (Rockville, MD 20852) in HB101 Escherichia coli. This plasmid confers tetracycline-resistance (but allows ampicillin-susceptibility) to HB101 E. coli. Plasmids are double-stranded self-replicating DNA particles like phages, but are native to the bacteria in which they reside and non-infectious to other species of bacteria. Restriction enzymes cut both DNA strands at (or near) their specific palindromic site. (Note: The "p" in pmEGF-26F12, pBR322 and pGEMEX stands for "plasmid.")

Because plasmids are double-stranded, they always contain sense (+) and antisense (-) complementary DNA. The antisense DNA produces the sense mRNA used by the host to make the specific protein. The sense DNA can be used to produce antisense RNA probes for in situ hybridization. An antisense cytokine RNA probe will hybridize specifically to the mRNA of its cytokine, whereas the sense probe will not.

Riboprobes are single-stranded, in contrast to cDNA probes, which are double-stranded. To use a double-stranded DNA probe for hybridization to mRNA, one must separate the two strands by heating (usually in 50% formamide). When used for in situ hybridization, some of the single-stranded cDNA probe will rehybridize with its complementary DNA mate, and some of the probe will hybridize with its specific mRNA. Therefore, single-stranded riboprobes usually work better than cDNA probes. cDNA probes, however, have proven to be satisfactory in other laboratories, as well as our own, for the in situ hybridization of IL-1 mRNA (see below).

Notes: (a) Sense means the 5' to 3' direction for the deoxyribose or ribose linkages in DNA or RNA. (b) Synthetic oligo-DNA probes are single-stranded like RNA probes. (c) There can be numerous copies of a given mRNA in the cytoplasm of a given cell, whereas the cell has usually only one copy of the DNA that produced the mRNAs.

E. coli (HB101) was grown in LB medium with tetracycline (12.5 ug/ml), according to methods described in Maniatis (1) on p. 88. The bacteria were collected by centrifugation, washed in NaCl-Tris-EDTA solution (ibid p. 89), and lysed with lysozyme, followed by sodium dodecyl sulfate (SDS) in weak NaOH (ibid p. 90). The solution was treated with 5 M potassium acetate buffer (pH 4.8), and centrifuged at 20,000 rpm (ibid p. 90). Large bacterial chromosome nucleic acids (NAs) and some proteins precipitate, while the plasmid (along with smaller DNA and RNA fragments and a few other proteins) remains in solution. The supernatant fluid was collected. The (plasmid) NA was precipitated at room temperature with 0.6 volumes of isopropanol, collected by centrifugation, washed with 70% ethanol, air-dried at 23°C with (or without) a "Speed Vac" concentrator (Savant Instruments, Inc., Farmingdale, NY), and suspended in Tris-EDTA buffer (ibid p. 91).

The plasmid DNA was purified on a $CsCl_2$ gradient at 55,000 rpm for 36 hr at 20°C (ibid p. 93). The purified plasmid was then cut with the Sph I restriction enzyme. pmEGF-26F12 is so constructed, that most of the EGF DNA insert (about 800 bp in size) can be excised by Sph I, and then inserted into the plasmid, pGEMEX. This we did, as described below. [The insert is not purified at this stage, but left as a mixture of insert and remaining plasmid.]

Lyophilized HB101 E. coli, containing the plasmid pGEMEX, was purchased from Promega Biotec (Madison, WI 53711), and grown in LB medium containing ampicillin (to which it is resistant). The pGEMEX was then purified by the methods just described. pGEMEX has only one Sph I site, so that Sph I was used to open the DNA ring. It also has a T7, a T3 and an SP6 promotor region for specific RNA polymerases which can make sense and antisense RNA from their respective DNAs (see below). The Sph I site is located between the T7/T3 and SP6 promotor sites.

T4 DNA ligase, from Bethesda Research Laboratories (Gaithersburg, MD 20877), was used to covalently link the excised EGF DNA fragment into the pGEMEX at the Sph I site (ibid p. 396). ATP is added to supply the energy to drive the reaction.

Since the EGF insert was not isolated from the plasmid, five types of pGEMEX constructs were made by the T4 ligase: (a) pGEMEX (the re-ligated original pGEMEX), (b) pGEMEX with the (sense) 800 bp EGF fragment inserted (now called pGEGF), (c) pGEMEX with the larger pmEGF-26F12 4000 bp fragment, (d) pGEMEX with

the 800 EGF bp fragment inserted in the antisense direction from the complementary DNA chain, and (e) pGEMEX with the larger pmEGF-26F12 4000 bp fragment also in the reverse direction. Constructs #2 and #4 are the desired products to be used to produce sense (+) and antisense (-) RNA probes, respectively, for the mRNA of EGF.

All five ligation products were inserted into E. coli (HB101) in the presence of $CaCl_2$ which makes the E. coli competent for (i.e., permeable to) the plasmid during the logarithmic phase of its growth cycle. Then, the E. coli was grown on ampicillin-containing agar plates, and 24 colonies were isolated, each of which would contain one of the five possible plasmid products.

These 24 colonies were expanded in LB broth (containing ampicillin), lysed, acetate-treated, and centrifuged (as described above). The supernatant fluids (containing the plasmid constructs' were collected and treated with the restriction enzyme, Sph I (or Sst I plus Hind III). The plasmids containing one of the two desired products (#2 and #4) showed 800 bp fragments on agarose gel electrophoresis. Three of the 24 E. coli colonies contained pGEMEX possessing the 800 bp EGF fragment. The remaining 21 clones contained the original pGEMEX with no inserts.

Note: In addition to these new pGEMEX constructs, there were similar constructs in the pmEGF-26F12 plasmid. However, the pmEGF-26F12 confers tetracycline resistance (not ampicillin resistance) to its E. coli host. (The pGEMEX confers ampicillin (not tetracycline) resistance to this host.) The bacterial colonies on the ampicillin plate, therefore, contained only the five types of pGEMEX plasmids described in the preceding paragraphs, and none of the E. coli containing original pmEGF-26F12.

We distinguished between the sense (#2) and antisense (#4) constructs by using the asymmetric Pst I site located within the 800 bp EGF sequence. For example, for the new type #2 EGF-pGEMEX construct, Sst I plus Pst I released a 500 bp fragment. Hind III plus Pst I released a 300 bp fragment. The Sst I site is adjacent to the "sense" T7 (RNA polymerase) promotor, whereas the Hind III site is adjacent to the "antisense" SP6 promotor. Therefore, the "sense" direction of the EGF insert produced the larger (500 bp) fragment. Conversely, when the EGF probe was inserted in the antisense direction (as in #4), the 300 bp fragment was released by Sst I plus Pst I, and the 500 bp fragment by the Hind III plus Pst I.

Sense or antisense pGEMEX-EGF constructs in E. coli were grown in LB broth, lysed, and the plasmids collected by centrifugation (as described above). The ring of the pGEMEX-EGF was opened at its single Hind III restriction enzyme-susceptible site (near the SP6 promotor site). Then, the T7 RNA polymerase was

used in the presence of ^{35}S-UTP and unlabeled ATP, CTP and GTP to make the antisense or sense RNA probes. Finally, the cDNA of the three plasmids was destroyed by DNase, leaving the sense and antisense RNA probes ready for use. For the production of these RNA probes, we used a kit purchased from Pharmacia LKB Biotechnology, Inc., (Piscataway, NJ 08854), which provided almost all of the reagents, except the ^{35}S-UTP.

The unlabeled ATP (an energy source) was present in excess. This T7 RNA polymerase was highly efficient: Abundant copies of sense or antisense RNA EGF probes for the mRNA were produced. Aliquots to be used in a week or so were stored at 4°C in Tris-EDTA (TE) buffer. Aliquots to be used later were stored at 4°C in 70% ethanol in TE buffer.

The antisense probes for the mRNAs of rabbit NAP-1 (IL-8), and rabbit MCP-1, rabbit IL-1 (beta), and rabbit TNF (alpha) were produced by methods similar to those just described for the human EGF probes. The DNA of these cytokines was purified from E. coli by the Alkaline Lysis Method, described on pp. 1.25 to 1.28 in reference (2), instead of the gradient method. Briefly, the E. coli were lysed with NaOH and sodium dodecyl sulfate. The bacterial debris (including their DNA) was precipitated with potassium acetate. After centrifugation, the plasmid DNA in the supernatant fluid was extracted with phenol-chloroform-isoamyl alcohol to remove the protein. The plasmid DNA was then precipitated with two volumes of ethanol at cold temperatures, washed with 80% cold ethanol, dried and resuspended in Tris-EDTA buffer. We then added pancreatic RNase A to digest the RNA fragments (pp. 1.51 and B.17 in ref. 2), and the phenol extraction and ethanol precipitation were repeated.

With these cytokines, we obtained both the antisense and sense probes from the same circular double-stranded DNA plasmid by cutting it with the appropriate restriction enzyme on opposite sides of the cytokine DNA insert and using either the T7 or the T3 promotor with the RNA polymerase (depending on how the cytokine DNA was inserted).

Note: We described, in detail, the molecular biology of preparing sense and antisense RNA for human EGF (a) because it was the first RNA probe that we made and (b) because it demonstrates how a cytokine (i.e., EGF) DNA insert is transferred from a plasmid used for cDNA probes to a plasmid (pGEMEX) that contains promoters for synthesizing RNA probes. Unfortunately, we were unable to demonstrate (human) EGF mRNA in (rabbit) SM lesions. Either, there is no cross-reactivity between human and rabbit EGF mRNAs, or there is no expression of EGF mRNA in SM lesions . . . or the conditions of the experiment were not adequate to demonstrate EGF mRNA.

In situ hybridization techniques

Unfixed and fixed frozen tissue sections of SM lesions were subjected to one of the following treatments:

Takacs Method for DNA probes (3). The slides were rehydrated in three sequential 10-min baths: 1) 5 mM $MgCl_2$ in 0.1 M sodium phosphate (pH 7.2)--0.15 M NaCl (PBS); 2) 0.1 M glycine--0.2 M Tris-HCl (pH 7.4); 3) 50% formamide--5X SSC (see page 6). Then, the slides were prehybridized for 4 hr at 37°C by using 10 ul of the following solution under a glass coverslip: 50% formamide, 1X Denhardt's solution (Ficoll- polyvinylpyrrolidone--bovine serum albumin, each 0.02% (1)), 1% glycine) 5X SSC, 50 mM sodium phosphate buffer (pH 6.5), 0.5 mg/ml E. coli transfer RNA (tRNA). Slides were rinsed in the third rehydration buffer (above). A probe mixture, consisting of the probe (about 200,000 cpm/slide for cDNA probes or 20,000 cpm/slide for oligo-DNA probes), salmon sperm DNA (250 ug/ml), yeast tRNA (85 ug/ml), formamide (50%) and SSC (2.8X), was boiled for 5 min, and cooled to about 60°C. Then, 50X Denhardt's solution was added to make a 1.5X final concentration. Ten ul of this probe mixture was placed on each tissue section. It was then covered with a glass coverslip, and hybridized for 20 hr at 37°C in sealed Petri dishes, containing filter paper moistened with the third rehydration buffer. Slides were washed in three sequential 30-min baths at 23°C: (a) 2X SSC, (b) 50% formamide 1X SSC, and (c) 1X SSC. Sections were prepared for autoradiography by using NTB-2 emulsion (Kodak), developed after 3 to 6 weeks, and stained with Giemsa.

Modified Modlin Method for RNA probes (4,5). Sections were digested at 37°C for 30 min in proteinase K (1 ug/ml of 100 mM Tris-HCl containing 50 mM EDTA at pH 8). Then, they were fixed for a second time for 10 to 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, (to stabilize cellular mRNA within the proteolyzed matrix). They were washed sequentially in 3X SSC, 1X SSC and 1X SSC, each for 5 min. After brief rinsing in DEPC-treated water (see p. 6), they were dehydrated sequentially in 30, 60, 80 and 95% ethanol and dried with a cool hair dryer. The free amino groups in the tissue sections were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min at 23°C. The sections were then rinsed briefly in DEPC-treated water and dried again with the hair dryer. For hybridization, the probe (denatured at between 80° and 95°C for 3 min) (just before use) was mixed with formamide (50% final concentration), NaCl (300 mM), Tris-HCl, (20 mM, pH 8.0), EDTA (5 mM), Denhardt's solution (1X), dextran sulfate (10%), dithiothreitol (DTT) (10 mM), and yeast tRNA (400 ug/ml). Ten ul of this hybridization solution containing 5 X 10^5 cpm probe was added to each slide. The tissue sections were covered with silicone-coated glass coverslips (previously baked at 150°C for 18 hr), which were then sealed with rubber cement around their edges. The sections were hybridized for 17-20 hr at 45°C in a moist chamber. The unhybridized probe was washed from the slides in a solution

of 50% formamide, 2X SSC, 10 mM DTT, and 1 mM EDTA for 30 min at 45°C. The sections were washed twice, briefly, with 2X SSC containing 10 mM DTT, and then digested with RNase A (20 ug/ml) for 30 min at 37°C. They were washed two more times with a solution of 50% formamide, 2X SSC, 10 mM DTT and 1 mM EDTA at 45°C for 30 min, each. In some experiments, a highly stringent wash of 0.2X SSC and 10 mM DTT was then used. Finally, the slides were briefly rinsed with a solution of 2X SSC and 1 mM DTT, dehydrated through graded ethanols containing 300 mM ammonium acetate, and then dried with the hair dryer. For autoradiography, slides were dipped into Kodak NTB-2 emulsion, diluted with equal parts of 600 mM ammonium acetate, and exposed at 4°C for 7-21 days. The slides were then developed and counterstained with Giemsa.

In situ hybridizations for cytokine mRNAs were performed with antisense probes (complementary to cellular mRNA). As negative controls, duplicate tissue sections were also hybridized with sense RNAs (homologous to cellular mRNA). Such positive and negative controls were included in each run.

PART B. REACTIVE OXYGEN INTERMEDIATES

Histochemical demonstration of hydrogen peroxide production in tissue sections (This method was adapted from references 6 to 11.)

Fixed and unfixed frozen sections were prepared as described above (on page 6). The slides containing the tissue sections were incubated for 5 to 18 hr at 37°C in 0.1 M HEPES buffer (pH 6.7), containing glucose (1.0 mg/ml), and 3,3'-diaminobenzidine tetrahydrochloride (1.0 mg/ml) (Sigma Chemical Co., St. Louis, MO 63178). The reaction product was not as dark at 5 hr as it was at 18 hr.

After incubation, the specific reaction product was intensified in 5% $CoCl_2$ in the HEPES buffer for 15 min at 23° C. The tissue sections were then rinsed in 0.9% NaCl, and, in some experiments, they were counterstained with Mayer's hematoxylin for 20 min at 23°C, in order to identify the cells more precisely. The sections were then dehydrated in 70, 95 and 100% ethanol, dipped in xylene, and mounted under a coverslip in Permount (Fisher Scientific Co., Fair Lawn, NJ 07410).

Hydrogen peroxide was identified as the main ROI produced by incubating duplicate tissue sections in the same reagent solution plus catalase (1.5 mg/ml) (Sigma). Catalase reduced the amount of specific staining to less than 10%.

RESULTS

PART A: CYTOKINES

Overview

About a year was spent getting the in situ hybridization method to work consistently. This method is known to be difficult and time consuming, but we never expected to have as much trouble with it as we had. [Other laboratories (that we consulted) have had similar difficulties.] Before he left Johns Hopkins to be a professor at Wayne State University, Dr. Fusao Hirata advised us on molecular biological techniques. Dr. Phoebe Mounts, an associate professor here at Hopkins, continues to advise us. She has had a great deal of experience with in situ hybridization procedures in tissue sections and has published several papers on these techniques. Dr. Mounts goes over the results of almost every experiment with us and helps us plan the next experiment.

The probes require high levels of radioactivity. The half-life of ^{35}S is 87 days, therefore, stocks must be replenished frequently. Also, over a period of several months, the probes auto-destruct because the ^{35}S irradiates the nucleotides. Finally, there is a relatively long waiting period before we know whether an experiment is successful: the autoradiography takes from one to three weeks in the dark room.

Nevertheless, the in situ hybridization technique is worth all the difficulties because it demonstrates microscopically which cytokines are produced by individual cells in tissue sections. In this sense, the technique is revolutionizing the field of experimental pathology, just as immunocytochemistry has done in the past. It enables the pathologist to identify cell functions in sections of tissue that is no longer viable.

Cytokine mRNA probes with which we have had limited (or no) success with in situ hybridization

Human oligoDNA (about 20 base-pair) ^{35}S probes for PLA_2 and IL-8 (provided by Dr. Fusao Hirata). With the Takacs method, PLA_2 mRNA was found to be present in macrophages, activated fibroblasts and granulocytes of nitrogen mustard lesions and 3-week rabbit dermal BCG lesions. BCG was used as a positive control for highly activated macrophages. No appreciable hybridization occurred with the IL-8 oligoDNA probe. We used nitrogen mustard instead of sulfur mustard for a good part of our first year, while waiting for our new hood with an activated carbon filter system to be built.

Human IL-1 (alpha and beta) ^{32}P-DNA (and ^{35}S-DNA) probes. We performed four initial experiments using these human IL-1 DNA probes with the Takacs method.

Several IL-1 (beta) mRNA--labeled macrophages were found in tissue sections of rabbit BCG lesions (our positive controls), but none were found in rabbit nitrogen mustard lesions. Since we had much better results with rabbit antisense RNA IL-1 probes (see below), we did not continue our work with this DNA probe.

Human EGF and human TNF ^{35}S-labeled antisense (and sense) RNA probes. Human EGF cDNA was received in plasmid pmEGF-26F12 from the American Type Culture Collection (ATCC 37486) in an E. coli. While working in our laboratory, Dr. Paul Hermonat transferred the EGF cDNA to pGEMEX, and made ^{35}S-labeled antisense and sense probes (see Experimental Methods).

Human TNF (alpha) cDNA was given to us in a pGEM plasmid by Dr. Bruce Beutler, of the University of Texas in Austin. From it, ^{35}S-labeled antisense and sense RNA probes were prepared in our laboratory.

Four experiments were performed on frozen tissue sections of rabbit dermal nitrogen mustard or sulfur mustard lesions, with BCG lesions as controls. None of the cells in these sections were labeled. Our laboratory now has rabbit ^{35}S-labeled TNF antisense and sense probes (see below). Therefore, our studies with human TNF probes on rabbit SM lesions will be discontinued. We shall, however, make fresh human EGF and TNF probes from these plasmids and use them on lesions produced in vitro by SM in discarded human skin. (See "Future Plans", below.)

Mouse ^{35}S-labeled GM-CSF, IFN (gamma), IL-2 and IL-4 RNA probes. Plasmids containing mouse GM-CSF and IFN (gamma) in pGEM-3 were given to us by Dr. Drew Pardoll of Johns Hopkins School of Medicine, and ^{35}S-labeled antisense (and sense) RNA probes were made in our laboratory. Mouse ^{35}S-labeled IL-2 and IL-4 probes were given to us (already made) by Dr. William Beschorner also of Johns Hopkins School of Medicine.

In one to three experiments with GM-CSF and IFN antisense RNA probes, frozen sections of 1-day mouse dermal nitrogen mustard lesions, rabbit dermal BCG lesions, and/or normal and BCG-infected mouse spleens showed no labeled cells. However, mouse thymus was labeled with probes for mouse IL-2, IL-4 and GM-CSF antisense RNAs in one experiment. [The sense RNA controls were negative.]

TNF (alpha). E. coli transfected with a Bluescript plasmid transformed with a rabbit TNF (alpha) cDNA insert was also given to us by Dr. Teizo Yoshimura (of Dr. Edward V. Leonard's group at the National Cancer Institute (NCI), of the National Institutes of Health, in Frederick, MD). We had great difficulty in preparing antisense and sense RNA probes from this plasmid because it contained contaminating DNA, possibly from a mutation. Subsequently, Dr. Yoshimura sent us a contaminant-free transfected pBluescript. However, in our two

experiments, we were not successful in demonstrating TNF (alpha) mRNA in tissue sections of SM lesions. Additional experiments are in progress.

Cytokine mRNA probes with which we have had much success with in situ hybridization

NAP-1 (also called IL-8) and MCP-1. These cytokines are produced by many cell types. NAP-1 is a major chemotactic and activating cytokine for granulocytes (and lymphocytes) (12-17). Although few lymphocytes are found in SM lesions, many PMN infiltrate SM lesions and are the predominant cells in their crusts. PMN proteases may contribute to the blister formation present in human SM lesions.

MCP-1 is a major chemotactic and activating cytokine for monocyte/macrophages. Macrophages are the predominant infiltrating cell type in peak SM lesions (19,20).

Dr. Teizo Yoshimura (of NCI in Frederick, MD), gave us E. coli JM101 transfected with pBluescript containing the DNA for rabbit NAP-1, and another E. coli strain transfected with pBluescript containing the DNA for rabbit MCP-1. In our laboratory, Dr. Junji Tsuruta and Kerry H. Bosley produced ^{35}S-labeled antisense and sense NAP-1 and MCP-1 mRNA probes from these pBluescript plasmids.

More than 12 experiments were performed with these probes for NAP-1 and MCP-1 mRNAs over the past year. Sulfur mustard lesions of various ages were evaluated, as well as a few nitrogen mustard lesions and BCG lesions. The first five experiments were unsuccessful, but we finally surmounted the difficulties, and have had uniformly successful results ever since.

Tables 1 and 2 present our findings. The activated macrophages and fibroblasts of acute and healing SM lesions contained high levels of NAP-1 and MCP-1 mRNAs (Fig. 1). Granulocytes contained lower levels of NAP-1 mRNA and hardly any MCP-1 mRNA. Granulocytes at the base of the crust showed more NAP-1 mRNA than did deeper granulocytes. Fewer cells in healing (6-day) lesions were labeled than in peak lesions. The sense RNA (control) probes did not label any cells.

In SM lesions, hair follicle cells (Fig. 2) were occasionally highly labeled with the NAP-1 and MCP-1 mRNA probes. The uneven distribution was probably due to variations in the penetration of SM. Only a rare hair follicle cell was labeled in normal skin with MCP-1, and none with NAP-1. A few surface epidermal cells were labeled with NAP-1, but not with MCP-1. The sense RNA (control) probes did not label any cells.

IL-1 (beta). Okayama plasmid containing rabbit IL-1 (beta) cDNA was given to us by Professor Masaru Yoshinaga, First Department of Pathology, Kumamoto University, Kumamoto, Japan. The IL-1 (beta) cDNA was excised and inserted into pBluescript by Dr. Yoshimura [of NCI]. Then, E. coli L-1 Blue, transfected with the recombinant pBluescript, was sent to us for expansion, isolation of the plasmid, and making the antisense and sense IL-1 (beta) RNA probes. Three successful in situ hybridization experiments were performed. The macrophage-fibroblast group and granulocytes of 1-, 2-, 3- and 6-day SM lesions were labeled for the mRNA for IL-1 (beta) (Table 1). No hair follicle cells or surface epidermal cells were labeled. Slightly fewer cells were labeled in healing (6-day) lesions than in peak lesions. The sense RNA (control) probes did not label any cells.

Distribution of cells with mRNA for these cytokines. NAP-1 mRNA was present mostly in the upper dermis within macrophages and fibroblasts from 2 hr to 6 days (Tables 1 and 2). It was also in granulocytes, but the amount of NAP-1 mRNA in PMN was usually less than that present in macrophages and fibroblasts. IL-1 mRNA has a distribution pattern similar to that of NAP-1 (studies in progress). MCP-1 mRNA was present in the macrophages and fibroblasts in the upper, middle and deep dermis. It was not present in PMN.

GRO. Dr. Yoshimura also sent us a recombinant pBluescript containing GRO cDNA, from which we produced ^{35}S-RNA probes. The GRO probes labeled many hair follicle cells, a moderate number of endothelial cells (of small blood vessels), and a somewhat smaller number of macrophage/fibroblasts. Granulocytes and surface epidermal cells were not labeled. GRO is a growth factor from the same family as NAP-1 (18,21). Actual counts on developing and healing SM lesions are still in progress.

PART B: REACTIVE OXYGEN INTERMEDIATES

Production of H_2O_2 in developing and healing sulfur mustard lesions

Our recently developed histochemical test showed that H_2O_2 was produced by the granulocytes (PMN) in the SM lesions. Two- and 3-day lesions showed peak numbers of PMN (19,20), and peak production of H_2O_2. In the dermis of healing (6-day) lesions, both the number of PMN and the amount of H_2O_2 they produced decreased substantially. In both early and late SM lesions, each intact PMN produced comparable amounts of H_2O_2. The crusts of healing (6-day) lesions were packed with live and disintegrating PMN (19,20). Most intact PMN, and many disintegrating PMN, produced H_2O_2 (Fig. 3).

In tissue sections, all cells are dead. The histochemical reaction product is produced by oxidative enzymes that are still active after death. The positive reaction in many of the disintegrating cells in the crust indicates that enzymes producing H_2O_2 *in vitro* also function in vivo for many hours after cell death.

We could not differentiate eosinophils from PMN in these frozen sections, but SM lesions only contain low percentages of eosinophils (19). [Rabbit PMN (really called heterophils) contain red-orange granules (22,23).] Some macrophages, and probably some activated fibroblasts (see 20), also produced H_2O_2.

Effects of fixation

We studied the effects of fixation on the H_2O_2 production demonstrated by this histochemical reaction. Mild fixation of the entire lesion (i.e., 4 hr at 4°C in 4% buffered paraformaldehyde -- see Experimental Methods) was highly effective in retaining the enzymes responsible for H_2O_2 production. The reaction product was over twice as intense after such mild fixation.

Inhibitors and activators of the reaction

Cells produce H_2O_2 by a variety of metabolic pathways (24). We, therefore, tested several components of these pathways to determine their effect on this histochemical reaction. Flavine adenine dinucleotide (FAD) (50 ng/ml to 500 ug/ml) had no appreciable effect. The reducing co-factors, NADPH and NADH (each 6.4 mM) inhibited the DAB reaction, but cysteine (0.5 mM to 5.0 mM), and glutathione (0.5 mM to 5.0 mM) had no appreciable effect. Catalase (0.15 mg/ml and above), which breaks down H_2O_2, inhibited the reaction, but superoxide dismutase (SOD) (200 to 1600 units/ml), which breaks down superoxide into H_2O_2 and water, had no effect.

In the literature, cerium chloride was used as a transition metal to initiate the polymerization of DAB (9,11), but our system, $CeCl_3$ (3.0 mM), had no effect on DAB polymerization. 3-Amino-1,2,4-triazole (200 mM), an inhibitor of myeloperoxidase (6), reduced the amount of reaction product. When we carried out the histochemical reaction in 95 to 100% oxygen, instead of in air, (using a sealed anaerobic-type jar), the brown DAB reaction product, as well as nonspecific background staining, were more intense.

The reaction product was also intensified by post-incubation of the tissue sections for 15 to 30 min at 23°C in 5% $CoCl_2$ in HEPES buffer (pH 6.7). This cobalt intensification procedure is, therefore, recommended for routine use.

Although studies to confirm many of the above results are still in progress, we tentatively conclude (a) that the histochemical reaction is specific for H_2O_2 production (b) that post-incubation in $CoCl_2$ enhances the color observed, and (c) that mildly fixed frozen sections are far superior to fresh frozen sections.

PART C: EXPERIMENTS WITH INFLAMMATORY INHIBITORS

Three recently available inflammatory inhibitors were tested for their effects on the development and healing of SM lesions. The first was Interleukin Receptor Antagonist Protein (IRAP) (25-27) from The Upjohn Company (Kalamazoo, MI). It binds to IL-1 receptors, but does not activate the cells possessing these receptors (28). It, therefore, acts as a competitive inhibitor for IL-1. IRAP is secreted by monocytes (macrophages) and keratinocytes (28) under the control of other cytokines (29-31) and other stimulants.

The second and third inhibitors tested were MK-886 (32) and L-656,224 (not published) from Merck-Frosst Centre for Therapeutic Research, in Pointe Claire-Dorval, Quebec, Canada. Both are leukotriene (LT) synthesis inhibitors. (LTB_4 is a major chemoattractant for granulocytes and monocytes.)

In rabbits, we injected these inhibitors (each 2.5 ug in 50 ul of 1% bovine serum albumin in 0.15 M NaCl) intralesionally, 5 hr after the topical application of 8 ul of 1% SM in methylene chloride. These injections were repeated daily for 3 more days. One rabbit was sacrificed on the fourth day. A second rabbit was similarly treated, but had an injection on the fourth day and was sacrificed on the seventh day. The lesions were measured daily, and histological sections were made of the lesions removed following sacrifice of the animal. (The two rabbits had 3 SM lesions, each, for IRAP, MK-886, L-656,224, the albumin diluent, and the untreated control SM lesions.)

IRAP had no detectable effect on the size of the lesions, on their rate of healing, or on the number and type of cells seen histologically. The two leukotriene inhibitors reduced slightly both the size of the lesions and the amount of cell infiltrate seen histologically, but healing did not seem to be accelerated.

This preliminary experiment suggests that these inhibitors are not promising candidates for the treatment of SM lesions. However, other dosages, routes and time schedules of administration might produce different results.

DISCUSSION

A. CYTOKINES

Cytokines are paracrine and autocrine hormones that activate or inhibit various cell functions in inflammatory areas. Most of the cytokines seem to be short-lived. For this reason, immunocytochemistry often, but not always (33-35), fails to demonstrate cytokine protein in tissue sections. Cytokine mRNA seems to be more stable than cytokine protein and can often be visualized in cells where the protein itself cannot be visualized.

Cytokines have mainly been studied in in vitro systems (36). The studies herein reported are among the few that attempt to assess the role of cytokines in vivo. Interpretation of in vivo results, however, is complicated by the redundancy of functions among the various cytokines (36). For example, IL-1 (alpha), IL-1 (beta), TNF and IL-6 have many overlapping functions (36), and synergism between them exists (37-39).

IL-1

The inflammatory response is, at least in part, initiated by cells producing IL-1 (17,28,40). IL-1 is a major cell activator (17,36,37). It stimulates the production of more IL-1 (37), and also other cytokines (39,40), e.g., IL-2, IL-3, IL-4, IL-6, TNF, GM-CSF, and IL-8, as well as prostaglandins (41,42). Many cells can produce IL-1 (36,37,40), e.g., macrophages, lymphocytes, fibroblasts, granulocytes, vascular endothelial cells, and keratinocytes.

More IL-1 (alpha) than IL-1 (beta) was found in normal human epidermis (43). However, in such epidermis, the amount of mRNA for IL-1 (alpha) is very low (43,44). IL-1 (alpha) is thought to be mainly cell-associated (28,36,44), whereas IL-1 (beta) is thought to be a soluble (secreted) mediator (28,36). IL-1 (beta) seems to be the major type produced by macrophages and many other cells (28,37). The role that IL-1 (alpha) and IL-1 (beta), as well as their receptor antagonist (IRAP) (see below), play in vivo in both normal and inflammatory conditions has been recently summarized by Bigler, et al. (28).

In SM lesions, we found high levels of mRNA for IL-1 (beta) in infiltrating macrophages and granulocytes, as well as in activated local fibroblasts, but none in epidermis. In human beings, immunocytochemical techniques showed an increase in the amount of IL-1 and TNF protein in epidermis after ultraviolet irradiation (UV) (33). We are planning similar immunocytochemical studies during the next 2 years, because the inflammatory processes produced by UV and SM have many common characteristics.

IL-1 increases the release of histamine from basophils (37,45). Therefore, it probably plays some role in the discharge of histamine from mast cells that followed the application of sulfur mustard to human skin explants (46). IL-1 also stimulates the synthesis of histamine (in hemopoietic cells) (47).

IL-1 is known to stimulate collagenase production (42,48) and, therefore, may play a role in the blister formation produced by SM in human beings. Finally, IL-1 may hasten the healing of SM lesions because topical recombinant IL-1 hastened the healing of skin wounds in pigs (49).

NAP-1 and MCP-1

Neutrophil attractant/activation protein 1 (or IL-8) and monocyte chemoattractant (and activating) protein 1 are major chemoattractant and activating cytokines for granulocytes and macrophages, respectively. NAP-1 also chemoattracts T lymphocytes (14,50,51) and basophils (50), but not eosinophils and monocytes (50). In fact, they are probably as important as the familiar leukocyte chemoattractants: complement C5a, FMLP (formylmethionylleucylphenylalanine), leukotriene B_4, and platelet activating factor. NAP-1 (12,13,) is produced by activated monocytes/macrophages, fibroblasts, lymphocytes, vascular endothelial cells, and keratinocytes (51). Three NAP-1 proteins have been identified, containing, respectively, 79, 77 and 72 amino acids (reviewed in 50). The 72 amino acid form is the most potent (52). MCP-1 (13,18) is produced by activated macrophages, fibroblasts, lymphocytes, vascular endothelial cells, smooth muscle cells, and keratinocytes (this report).

In SM lesions (but not in normal skin) NAP-1 mRNA was found in both epidermal and hair follicle keratinocytes, and MCP-1 mRNA was found in hair follicle keratinocytes. These findings suggest that the production of these two chemoattractants was up-regulated by the irritation produced by SM. The highest levels of SM are on the surface, whereas lower levels reach the hair follicle keratinocytes. The surface cells slowly die over the next 8 to 12 hours, but most of the follicle cells remain viable. Thus, the stimulation produced by SM on surface cells is short-lived -- which may explain why the surface NAP-1 mRNA was at low levels, and surface MCP-1 mRNA was not detected. In contrast, the mRNA of both cytokines up-regulated in hair follicle cells of SM lesions. Like IL-1, NAP-1 releases histamine from basophils (53,54) but, in one investigation, did not cause mast cell degranulation (55). MCP-1 is the most potent histamine-releasing cytokine found to date (56). It released histamine from basophils (56), but its effect on mast cells remains to be evaluated.

Other cytokines

GRO (21,57-59) is a member of the proinflammatory "intercrine" cytokine family (listed in 18,21). It includes MCP-1(JE) RANTES, NAP-1 (IL-8), PF-4 and others. GRO and some of its components are also chemotactic and probably play a role in the inflammatory response. TNF (alpha) is a broad-spectrum inflammatory cytokine, like IL-1 and IL-6, with overlapping activities (17). TGF (beta) is a major regulator of cytokine activity (60,61). We have or are developing ^{35}S-antisense RNA probes for these three cytokines.

B. REACTIVE OXYGEN INTERMEDIATES

Hydrogen peroxide production

Catalase, which specifically hydrolyzes H_2O_2 into H_2O and O_2, greatly reduced the brown color produced histochemically in the cells of SM dermal inflammatory lesions. Therefore, H_2O_2 seemed to be the main reactive oxygen intermediate (ROI) that oxidized and polymerized the diaminobenzidine (DAB) substrate.

Diaminobenzidine is used histochemically to demonstrate a variety of oxidases (11). In these reactions, (a) the exogenous specific substrate for the oxidase, (b) DAB, and (c) exogenous H_2O_2 are present in the reagent solution, and the reaction is usually carried out at 23°C for less than 1 hr. In our histochemical reaction, the reaction is carried out for 5 to 18 hr at 37°C. No specific substrate or H_2O_2 is added, but glucose is supplied as the energy source. Therefore, all of the oxidant substrates and enzymes required to polymerize DAB are present in the tissue section itself.

Is DAB bound nonspecifically to certain cells (e.g., granulocytes and macrophages) and directly oxidized by the dissolved oxygen in the incubating solution? Since the specific reaction was abolished by the addition of catalase, direct oxidation by dissolved oxygen seems to be ruled out. The background nonspecific color, however, was intensified by incubating in 95 to 100% oxygen, rather than air, indicating that some DAB is slowly oxidized by dissolved O_2 during this incubation time. Such mild oxidation creates no problem because the specific reaction caused by H_2O_2 production is much more intense.

During the 5- to 18-hr incubation at 37°C, the H_2O_2 needed to polymerize DAB was produced by oxidases already present in the tissue section without the exogenous addition of any substrate but glucose. There are a variety of these oxidases, the most plentiful of which are used to carry out cell respiration, e.g., NADH oxidases and NADPH oxidases (7,24). Others, such as xanthine oxidase,

D-amino acid oxidase, monoamine oxidase and alpha-hydroxy acid oxidase also produce H_2O_2 (11).

Once the H_2O_2 was formed, a peroxidase is needed to catalyze the oxidation of DAB to the insoluble brown reaction product (62). Granulocytes and activated macrophages contain large amounts of peroxidases. In fact, in a recent experiment, we added H_2O_2 to the reaction mixture and the amount of the reaction product doubled. Thus, the production of H_2O_2, not the amount of peroxidase, seems to be rate limiting. We are repeating this experiment to confirm these findings, and also performing experiments to determine whether the histochemical reaction requires the addition of glucose. In vivo, glucose levels are usually adequate and are maintained under careful control.

Catalase (0.15 to 1.5 mg/ml) was our most specific control. It eliminated the positive DAB reaction product in PMN and in the macrophage-fibroblast group. However, catalase apparently reduced, but did not eliminate, the positive reaction product in eosinophils. Eosinophils, therefore, probably produce much more H_2O_2 than do PMN.

Tissue damage by ROIs

No tissue damage was found adjacent to cells producing ROIs. Therefore, the ROIs must be in non-toxic concentrations, or they must be rapidly inactivated soon after they are formed. Cells and extravasated serum protect tissues from oxidant damage in many ways (63). Cells contain SODs, catalase and peroxidases, e.g., myeloperoxidase, and selenium-containing glutathione peroxidase. Serum contains antioxidants, such as ceruloplasmin and albumin (a major sacrificial antioxidant (64), i.e., one that is oxidized itself, sparing more vital host components). Also tocopherol (vitamin E), ascorbic acid (vitamin C), beta-carotine (a precursor of vitamin A) are micro-nutrient antioxidants (63). Only when these "shields" are inadequate does local damage occur.

Thus, it was not surprising that no evidence of tissue damage was found adjacent to cells producing H_2O_2 in the dermal SM lesions. Such lesions contain large amounts of extravasated serum (19,65,66). Evidently, the oxidants produced by the phagocytes remain attached to their lysosomal (and plasma) membranes (presumably for antimicrobial defense) and do not cause any histologically recognizable damage to host tissues. Even under the lesion crust, which produces major amounts of H_2O_2, the tissues appear viable. In fact, during healing, keratinocytes readily grow under the crust, from the edge of the wound (and the hair follicles), replacing the damaged epithelium (19).

C. INHIBITORS OF THE INFLAMMATION PRODUCED BY SM

Interleukin 1 receptor antagonist protein (IRAP), also abbreviated IL-1ra, (25-31,67-69), is one of the first specific cytokine inhibitor available for clinical trials. It was isolated, cloned and produced almost simultaneously by both Upjohn (25) and Synergen (26,27,68), both of which were happy to supply samples for us to evaluate. [We chose Upjohn because they were the first to answer our request, and I had met Dan Tracy (who answered our request) while he was in training here at Hopkins.]

IRAP is a member of the IL-1 gene family (68). It has a classic secretory leader peptide and is glycosylated. It blocks the effects of IL-1 (alpha) and IL-1 (beta) by binding to the IL-1 receptor. (A single receptor binds both forms.) IL-1 is present in high amounts in normal human skin without any sign of inflammation (69). This is apparently because keratinocytes contain IRAP (IL-1ra). Differentiated keratinocytes contain more IRAP than do non-differentiating cells. Thus, the lack of inflammatory response in normal skin (which contains large amounts of IL-1 (alpha) (43) is probably due to the high levels of IRAP present.

Our first experiment showed that IRAP had no effect on the development and healing of SM lesions. [The leukotriene inhibitors MK-886 and L-656.224, from Merck Frosst, did decrease the severity of the SM lesions, but the effects were not pronounced.] Because IL-1 is an important initiator of the inflammation that follows irritation and injury, we plan to carry out more studies with IRAP at a higher dosage level.

Human skin

Our antisense probes for human TNF (alpha) mRNA and human EGF mRNA did not hybridize in tissue sections of rabbit SM lesions. We shall, therefore, use these probes on tissue sections of discarded human skin (from surgical operations) that have been exposed to SM in vitro and organ-cultured for 24 hr. (See Future Plans, below.)

FUTURE PLANS

1. To continue to perfect our in situ hybridization techniques for the various cytokines mentioned in this report. The main challenge remaining is to obtain good results in paraffin- or Immunobed-embedded tissues. If successful, the cells in such tissue sections will be identified more precisely than in frozen sections. The data in Table 1, for NAP-1 and MCP-1, were collected by painstaking, time consuming cell counting in one experiment. These data need to be confirmed by counting the cells in other experiments. Such meticulous counting of cells containing the mRNA of other cytokines must also still be performed.

2. To study the effects of lower concentrations of SM. To date, except for NAP-1 mRNA, we have not been able to demonstrate cytokine mRNA in the surface epidermal cells of sulfur mustard lesions. Perhaps, the injury by SM was too great.

3. To evaluate additional probes for cytokine mRNA, e.g., TGF (beta), a major regulator of inflammatory cell function. We hope to include GM-CSF (which stimulates PMN and monocyte production in the bone marrow and activates these cells), IL-3 (a mast cell growth factor), and some single-stranded oligo-DNA and oligo-RNA probes.

4. To avoid the problem of cross-species unreactivity, we shall begin some human skin experiments. Specifically, we shall obtain discarded human skin from mastectomies and various plastic surgical procedures. The skin is cut into 1.0-cm^2 pieces, topically exposed to SM in vitro, and organ-cultured for 18 hr, along with $MeCl_2$-exposed controls. (See the final report of our previous contract DAMD17-87-C-7040.) The purpose of these studies is to identify the cytokines produced by epidermal cells exposed to SM. These cytokines could initiate the inflammatory response produced by this vesicant. We have been approved (as "Exempt") for such human skin studies by our Institutional Review Board and also, recently, by the Army.

5. To perform more studies with immunocytochemistry for cytokine proteins in sulfur mustard lesions. We have obtained good preliminary results in rabbit lesions with an antibody to swine TGF (beta) and the avidin-biotin-peroxidase immunocytochemical technique. The TGF was present in the epidermal cells and hair follicle cells of SM lesions. Whether or not normal epidermal cells must be activated by the inflammatory process to produce TGF (beta) remains to be evaluated. We have obtained goat antibody to rabbit IL-1 (alpha) and IL-1 (beta) for additional immunocytochemical studies. [IL-1 (alpha) is known to be present in epidermal cells; whereas IL-1 (beta) is known to be present in macrophages.]

These immunocytochemical studies should identify cells that contain the protein of a given cytokine. In situ hybridization only demonstrates cells that contain the cytokine mRNA code. Cells with receptors that have bound to cytokine would also be identified by immunocytochemical procedures.

6. To study the effects of IRAP, MK-866 and L-565-224 on the development and healing of sulfur mustard lesions, using 10 times the local dosage that we previously used. We also hope to obtain new experimental antiinflammatory agents (especially existing cytokine inhibitors, when available), in order to test their effects on rabbit dermal sulfur mustard lesions.

7. To prepare for publication our studies on developing and healing sulfur mustard lesions with our newly developed histochemical test for cells producing hydrogen peroxide.

8. To prepare for publication our studies on cytokine mRNAs and cytokine protein in developing and healing SM lesions.

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Dr. Phoebe Mounts, Associate Professor of Immunology and Infectious Diseases, advised us weekly on the molecular biology of the cytokines herein.

Dr. Fusao Hirata, (formerly of our department here at the Johns Hopkins School of Hygiene, now at Wayne State University), a subcontractor on our Contract, produced the initial human IL-1 (alpha), Il-1 (beta) and TNF (alpha) cDNA probes, and also the human IL-8 and PLA_2 oligo-DNA probes.

Dr. Paul L. Hermonat, who worked for several months as a Research Associate on our Contract, produced the EGF antisense and sense RNA probes.

Dr. Edward J. Leonard and Dr. Teizo Yoshimura, of the National Cancer Institute of NIH (in Frederick, MD), provided plasmids for every rabbit cytokine that we used [NAP-1 (IL-8), MCP-1, IL-1 (beta) and TNF (alpha)]. Without their help, we would not have been so successful in the studies herein reported.

Figure 1: MCP-1 mRNA in a rabbit 3-day dermal sulfur mustard (SM) lesion. SM evidently caused cells in the macrophage/fibroblast group to produce MCP-1 mRNA. In normal skin, very few cells label with the MCP-1 RNA probe. ^{35}S-sense MCP-1 RNA probes did not label any cell. (An autoradiograph of a frozen section of cold-formalin-fixed tissue hybridized in situ with ^{35}S-labeled antisense MCP-1 RNA, counterstained with Giemsa. X 500)

Figure 2: NAP-1 (IL-8) mRNA in a rabbit 3-day dermal SM lesion. SM evidently caused the keratinocytes in this hair follicle to produce much NAP-1 mRNA, because very few hair-follicle cells in normal skin label with the NAP-1 probe. ^{35}S-sense NAP-1 RNA probes did not label any cell. (An autoradiograph of a frozen section of cold-formalin-fixed tissue hybridized in situ with ^{35}S-labeled antisense NAP-1 RNA, counterstained with Giemsa. X 500)

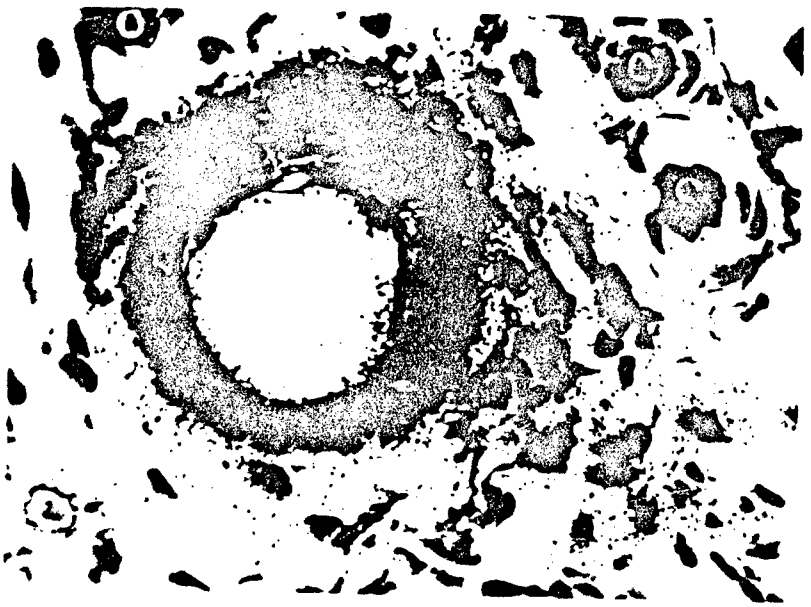

Figure 3: H_2O_2 production in a tissue section of a rabbit 3-day dermal mustard lesion. Intact granulocytes in the tissues and in the crust oxidized the diaminobenzidine substrate, producing an insoluble brown polymer. Recently disintegrating granulocytes in the crust also oxidized DAB. Catalase (0.15 to 1.5 mg/ml) inhibited the reaction, identifying H_2O_2 as the oxidant of the DAB. (Frozen section of cold-formalin-fixed tissue, incubated at 37°C for 16 hr in DAB and glucose at pH 6.7, followed by intensification in $CoCl_2$ for 30 min; no counterstain. X 500)

Table 1: Cytokine mRNAs in developing and healing SM lesions: In situ hybridization data showing which cells produce which cytokines and when*

Cell Type	Age of SM Lesions								
	NAP-1								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts	-	±	++	++	+++	+	+++	++	+
Neutrophils	-	-	-	+	+	±	+++	+	+
Surface epidermal cells	-	-	±	-	±	±	-	±	-
Hair follicular cells	-	±	++	+	++	+	±	+	±
	MCP-1								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts	±	+	+++++	+++++	+++	+++++	++++	++++	++++
Neutrophils	-	-	-	-	-	-	-	-	-
Surface epidermal cells	-	-	-	-	-	-	-	-	-
Hair follicular cells	±	-	±	±	-	±	-	-	-

continued . . .

Table 1: Cytokine mRNAs in developing and healing SM lesions: In situ hybridization data showing which cells produce which cytokines and when* (cont'd)

Cell Type	Age of SM Lesions								
	IL-1 (beta)								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts	-	±	±	+	++	+++	+++	++	++
Neutrophils	-	-	-	±	+	++	+++	++	++
Surface epidermal cells	-	-	-	-	-	-	-	-	-
Hair follicular cells	-	-	-	-	-	-	-	-	-

*Cold (4°C) 4% paraformaldehyde-fixed frozen tissue sections of SM lesions were hybridized with ^{35}S-labeled antisense RNA probes for rabbit NAP-1, MCP-1, and IL-1 (beta). ^{35}S-labeled sense RNA probes were our negative controls.

Labeled-cell counts were made microscopically in superficial, mid and deep areas of the skin (see Table 2). The crust (scab) area was not included. Forty grids (0.25 mm X 0.25 mm) were counted in each of the three areas. The tissue sections were 1.0 ±0.2 cm long. Counts were expressed as cells per mm^2 of tissue section. ± = 1 to 10 cells labeled; + = 10 to 40 cells labeled; ++ = 40 to 80 cells labeled; +++ = 80 to 160 cells labeled; ++++ = 160-240 cells labeled; +++++ = 240 to 320 cells labeled. In general, the average number of silver grains per cell increased appreciably as the number of labeled cells per mm^2 increased, but no quantitation of the grain counts was made. The IL-1 (beta) data (above) are estimates only, as we are still counting the cells in tissue sections hybridized with the IL-1 antisense RNA probes.

Conclusions from these data: A greater percentage of cells contained cytokine mRNA in peak (1- to 2-day) lesions than in healing (6-day) lesions. Granulocytes (mostly PMN) contained NAP-1 mRNA and IL-1 mRNA, but not MCP-1 mRNA. Cells in the macrophage-fibroblast group were labeled for all three cytokines. Surface epidermal cells only labeled for NAP-1 mRNA. Hair follicle cells were labeled for NAP-1 and MCP-1 mRNAs. [SM penetrates into many hair follicles because the hair shaft has a high lipid content.] Note: The dead (PMN) cells of the crusts of healing lesions contained NAP-1 mRNA and IL-1 mRNA, but not MCP-1 mRNA (not presented in this table).

Table 2a: Distribution (in rabbit SM lesions) of granulocytes that hybridize with NAP-1 antisense RNA*

	Normal skin			Peak lesions (1&2 day)			Healing lesions (3&6 day)		
Skin depth	Negative cells/ mm^2	Total labeled cells/mm^2	%	Negative cells/ mm^2	Total labeled cells/mm^2	%	Negative cells/ mm^2	Total labeled cells/mm^2	%
Upper	3	0	0	1 da/242	1.2	0.5	3 da/678	25	3.6
				2 da/654	76	10.4	6 da/403	12	2.9
Middle	1	0	0	1 da/ 99	0.4	0.4	3 da/ --	--	---
				2 da/617	10	1.6	6 da/ 33	0	0
Deep	1	0	0	1 da/ 60	0	0	3 da/ --	--	---
				2 da/828	5	0.6	6 da/ 13	0	0
Total per 3 mm^2	5	0	0	1 da/401	1.6	0.4	3 da/ --	--	---
				2 da/2099	91	4.1	6 da/449	12	2.6

* Legend is the same as that for Table 1

Table 2b: Distribution (in rabbit SM lesions) of macrophage/fibroblast cells that hybridize with NAP-1 antisense RNA*

	Normal skin			Peak lesions (1&2 day)			Healing lesions (3&6 day)		
Skin depth	Negative cells/ mm^2	Total labeled cells/mm^2	%	Negative cells/ mm^2	Total labeled cells/mm^2	%	Negative cells/ mm^2	Total labeled cells/mm^2	%
Upper	1293	0	0	1 da/787	26	3.2	3 da/1068	72	6.3
				2 da/583	139	19.3	6 da/590	39	6.2
Middle	525	0	0	1 da/432	2	0.5	3 da/ --	--	---
				2 da/1021	4	0.4	6 da/510	0	0.0
Deep	343	0	0	1 da/584	1	0.2	3 da/ --	--	---
				2 da/1709	2	0.1	6 da/425	0	0.0
Total per 3 mm^2	2161	0	0	1 da/1803	29	1.6	3 da/ --	--	---
				2 da/3313	145	4.2	6 da/1525	39	2.5

* Legend is the same as that for Table 1

Table 2c: Distribution (in rabbit SM lesions) of macrophage/fibroblast cells that hybridize with MCP-1 antisense RNA*

	Normal skin			Peak lesions (1&2 day)			Healing lesions (3&6 day)		
Skin depth	Negative cells/ mm^2	Total labeled cells/mm^2	%	Negative cells/ mm^2	Total labeled cells/mm^2	%	Negative cells/ mm^2	Total labeled cells/mm^2	%
Upper	1076	3	0.3	1 da/620	27	4.2	3 da/720	72	9.1
				2 da/578	23	3.8	6 da/1045	154	12.8
Middle	308	0	0	1 da/217	150	40.9	3 da/460	86	15.8
				2 da/264	116	30.5	6 da/508	10	1.9
Deep	280	0	0	1 da/228	91	28.5	3 da/481	50	9.4
				2 da/269	93	25.7	6 da/372	7	1.8
Total per 3 mm^2	1664	3	0.2	1 da/1065	268	20.1	3 da/1661	208	11.1
				2 da/1111	232	17.3	6 da/1925	171	8.2

* Legend is the same as that for Table 1

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