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Reduction of Intestinal Carcinogen Absorption by Carcinogen-Specific Secretory Immunity

LAWRENCE K. SILBART AND DAVID F. KEREN

A secretory immune response to the carcinogen 2-acetylaminofluorene (AAF) was elicited in rabbits by directly immunizing the small intestine with an AAF–cholera toxin conjugate. High-titer, high-affinity secretory immunoglobulin A (IgA) antibody to AAF was secreted into the intestinal lumen in response to this immunogen. Immune secretions reduced the transepithelial absorption of a ^{125}I-labeled derivative of AAF by more than half. This reduction of absorption by hapten-specific IgA suggests that oral vaccines against carcinogens and toxicants could be developed for humans.

PATHOGENIC ORGANISMS, BACTERIAL toxins, and environmental toxicants often enter the mammalian body by traversing a mucosal surface. A major mechanism to block transport at these surfaces involves the secretion of antigen-specific immunoglobulins of the IgA isotype. The protective effect conferred by the mucosal immune system is illustrated in humans by the success of a recent oral cholera vaccine containing cholera toxin B subunit and heat-killed *Vibrio cholerae* and providing immunity to the disease (*1, 2*).

We now show a mucosal immune response in rabbits to the chemical carcinogen AAF, which had a high-affinity, high-titer luminal IgA component that reduced the absorption of a radioiodinated derivative of the compound in vivo. When AAF is administered orally it is absorbed across the gastrointestinal mucosa into the blood stream and becomes a substrate for various metabolic reactions. Some metabolic pathways activate the chemical into highly reactive electrophilic intermediates capable of covalently binding to cellular macromolecules (*3, 4*). Covalent modification of nuclear DNA is believed to be an initiating event in the development of chemically induced cancer (*3, 5*).

Although a humoral immune response (IgG) can be elicited on exposure to some carcinogens (*6*), this response is substantially enhanced by covalently attaching the molecule to a foreign carrier protein (*7*). To elicit a mucosal immune response, we prepared conjugates of AAF covalently coupled to the known mucosal immunogen cholera toxin (*8, 9*). The AAF–cholera toxin (AAF-CT) mucosal immunogen was prepared by a modification of the methods published for covalently linking *N*-2-fluorenyl succinamic acid (SAF) to carrier protein (*10, 11*). The resulting conjugates had low substitution ratios (8 to 13 SAFs per CT); however, they retained their ability to bind the ganglioside GM_1 [the surface epithelial receptor for cholera toxin (*12*)]. In addition, the AAF-CT conjugates maintained their mucosal immunogenicity with respect to both AAF and cholera toxin.

The isolated rabbit ileal (Thirty-Vella) loop model (*13*) was used to monitor the secretory IgA response to AAF-CT conjugates. We collected daily intestinal secretions before and after immunizations. A vigorous antibody response to AAF was observed in the intestinal secretions collected from all immunized rabbits, with material obtained from three of four rabbits binding more than 10% of an iodinated radiotracer [^{125}I-labeled *N*-2-(4-hydroxyphenyl)acetamidofluorene or ^{125}I-pHP-AAF (*10*)] offered at titers of 10^3 (nonspecific binding to nonimmune secretions was less than 2%). The epitope and isotype specificity of the mucosal immune response was determined by competitive enzyme-linked immunosorbent assay [ELISA (*10*)]. In these studies, incubation of 1:20 dilutions of ileal loop secretions in the presence of AAF inhibited binding to the AAF–carrier protein conjugates in a concentration-dependent manner (Fig. 1), indicating antibody recognition of the AAF epitope on the AAF-CT conju-

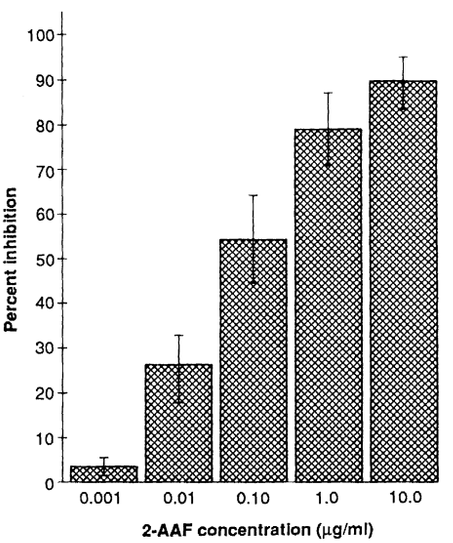

Fig. 1. The IgA anti-AAF isotype and epitope specificity were determined in rabbit intestinal secretions by competitive ELISA studies as described (*10*) with goat antibody to rabbit IgA (affinity purified) conjugated to alkaline phosphatase [GARA (*14*)]. The AAF was preincubated with 1:20 dilutions of immune secretions for 1 hour at room temperature. A 100-μl sample was applied in duplicate to microtiter wells previously coated with either AAF–bovine serum albumin (BSA) conjugates, BSA alone, or uncoated wells. After a 4-hour incubation at room temperature, the plates were washed and treated with GARA overnight. After washing, the *p*-nitrophenyl phosphate (1 mg/ml) was added, and the absorbance was measured (405 nm) after 100 min. The percent inhibition was calculated on the basis of 0% for wells containing no AAF. Values represent the mean inhibition ± SE (n = 4) after subtraction of absorbance in the corresponding BSA wells (nonspecific binding). AAF had no effect on unrelated antigen-antibody interactions over the range of concentrations tested (*15*). Since the AAF stock solution was prepared in dimethylformamide (DMF), DMF control (5% and 0.5%) incubations with the uninhibited immune secretions were included on each plate and used in the appropriate calculations. The secretory immune response to AAF was elicited by the following regimen: four rabbits received 100 μg of the AAF-CT conjugate in 1 ml of phosphate-buffered saline (PBS) directly into a chronically isolated ileal loop approximately 3 hours after the ileal loop surgery. Identical booster doses were given on days 7, 14, and 21 after the surgery and priming dose. The immune response of each rabbit was initially measured by the binding of an ^{125}I derivative of AAF in a radioimmunoassay method as described (*10*).

University of Michigan, Pathology Department, Ann Arbor, MI 48109.

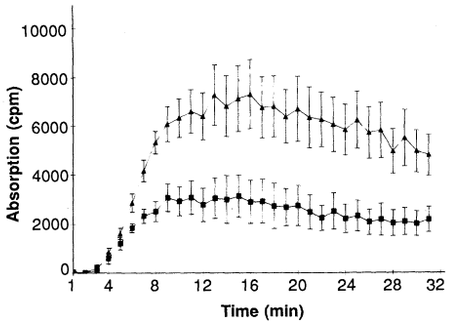

Fig. 2. The transepithelial absorption of ^{125}I-pHP-AAF (*10*) was estimated by measurement of the gamma emissions contained in each 1-min sample of mesenteric blood (*17*). The absorption profile from intestinal sections injected with either 1 ml of immune secretions (■) or 1 ml of nonimmune secretions (▲) is shown. Values are the means ± SE (n = 5).

gates. The IgA isotype of these anti-AAF immunoglobulins was confirmed by use of affinity-purified goat antibody to rabbit IgA conjugated to alkaline phosphatase [GARA (*14*)] in the ELISA system. When purified IgA from an unimmunized animal was incubated with GARA, its binding to IgA specific for AAF was completely inhibited (*15*). Ileal loop secretions from each of two immunized rabbits were separately pooled, and the IgA fraction from each pool was isolated (*14*). The average affinity constant for AAF was estimated (*16*) from each pool, with $K_{av} = 1.0 \times 10^9$ and $2.2 \times 10^9 M^{-1}$.

To determine whether immune secretions reduced absorption of the AAF from the rabbit intestinal lumen to the mesenteric blood, we used the ^{125}I-pHP-AAF radiotracer (*17*). Although this tracer is more polar than the parent carcinogen, the affinity of the antibody toward the tracer was within one order of magnitude of its affinity toward AAF and should model the behavior of the parent carcinogen quite closely. A dramatic reduction in the amount of derivatized carcinogen reaching the bloodstream was observed in the section of intestine receiving the immune secretions as compared to the section receiving nonimmune secretions (Table 1). The transepithelial absorption of the derivatized carcinogen in the presence and absence of immune loop secretions is shown in Fig. 2. The absorption of label into the mesenteric blood was more than twice as high in the nonimmune loop sections as in the passively immunized section (Table 1). Conversely, a larger amount of the label remained in the immunized loop section contents, with a large percentage bound to macromolecules (presumably anti-AAF immunoglobulins). We infer that the label was bound to macromolecules and prevented absorption (Table 1). Additional support for this inference was observed when 1.0 ml of purified IgA specific for AAF (0.84 mg/ml) was included in the loop section of an unimmunized rabbit. The distribution of radioactivity observed in this study was similar to that observed with the immune secretions (Table 1).

These findings are consistent with other observations on the mucosal immune system. Antibody (IgA) against hapten groups is elicited after appropriate antigen priming (*18*). For instance, when four intraperitoneal doses of *N*-acetyl glucosamine (GlcNAc) conjugated to hemocyanin (Hy), followed by an intraduodenal dose of GlcNAc conjugated to cholera toxin is given to mice, a high percentage of Peyer's patch B cell clones that express the IgA isotype are specific for GLcNAc. Furthermore, oral immunization of rats with the antigen horseradish peroxidase (HRP) results in a decrease in intestinal absorption of HRP when the in vitro everted gut sac model is used (*19*). Gel diffusion studies demonstrated the presence of antibodies to HRP in secretions and mucosal extracts from the immunized animals.

Moolten *et al.* (*20*) first suggested that the development of a mucosal immune response to a carcinogen could facilitate excretion of the compound by preventing its absorption into the body. Mice were immunized with an intraperitoneal dose, which was supplemented twice by an intragastric dose of a fluorinated derivative of 7,12-dimethylbenzanthracene (DMBA) covalently coupled to bovine serum albumin. When animals were given a challenge exposure of [^{3}H]benzo(*a*)pyrene (a closely related carcinogen expected to cross-react with antibodies to DMBA), more tritium was found in the γ-globulin fraction of fecal extracts of immunized mice than in nonimmunized control mice. No direct determination of the titer, isotype, or affinity of these antibodies was published, nor have there been any subsequent reports regarding the capacity of the secretory immune system to interfere with gastrointestinal carcinogen absorption.

Although the rabbits used in the transepithelial uptake studies were passively immunized, these studies demonstrate that the initial phase of absorption of the derivatized carcinogen could be substantially reduced by the presence of intraluminal secretory immunoglobulins specific for the carcinogen. These findings may have broad implications for the ability of the mucosal immune system to abrogate the effects of other carcinogens and toxicants (for example, food mutagens and environmental contaminants such as aflatoxin B_1). The development of effective oral vaccines may provide a convenient mechanism by which absorption of these toxicants can be prevented.

Table 1. Distribution of ^{125}I-pHP-AAF recovered after short-term loop surgery (*17*). The values are the percentage of the total recovered label (mean ± SD) remaining in each compartment at the conclusion of the 31-min period of observation. In the immune secretions groups, rabbits had 1 ml of high-titer anti-AAF ileal loop secretions (from a rabbit immunized with an AAF-CT conjugate) injected into their acute loop section. In the nonimmune secretions group, rabbits had 1 ml of nonimmune secretions (from a rabbit immunized with an unrelated immunogen, *Shigella flexneri* X16) injected into the short-term loop section. In the purified immune secretory IgA group, rabbits received 0.84 mg of purified IgA (from the ileal loop secretions of an immunized rabbit) injected into the short-term loop section.

Group	% Recovered ^{125}I-pHP-AAF remaining in			
	Loop fluid		Loop tissue	Blood
	Total	Bound*		
Immune secretions (n = 5)	81 ± 2.8†	(83 ± 2.4)†	12.6 ± 3.7†	6.3 ± 2.6‡
Nonimmune secretions (n = 5)	52 ± 4.3	(14 ± 5)	33.4 ± 5.1	14.9 ± 3.7
Purified immune IgA	86	(79)	5.3	9

*The bound counts are the percentage of the total radioactivity present in the residual loop fluid bound to macromolecules [as determined after separation by means of dextran-coated charcoal (*10*)]. †Statistically different from the nonimmune group at $P < 0.001$ (Student's t test). ‡Statistically different from the nonimmune group at $P < 0.005$ (Student's t test).

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The Neutrophil-Activating Protein (NAP-1) Is Also Chemotactic for T Lymphocytes

CHRISTIAN GRØNHØJ LARSEN, ARTHUR O. ANDERSON, ETTORE APPELLA, JOOST J. OPPENHEIM, KOUJI MATSUSHIMA*

T lymphocyte chemotactic factor (TCF) was purified to homogeneity from the conditioned media of phytohemagglutinin-stimulated human blood mononuclear leukocytes by a sequence of chromatography procedures. The amino-terminal amino acid sequence of the purified TCF showed identity with neutrophil-activating protein (NAP-1). Both TCF and recombinant NAP-1 (rNAP-1) were chemotactic for neutrophils and T lymphocytes in vitro supporting the identity of TCF with NAP-1. Injection of rNAP-1 into lymphatic drainage areas of lymph nodes in Fisher rats caused accelerated emigration of only lymphocytes in high endothelial venules. Intradermal injection of rNAP-1 caused dose-dependent accumulation of neutrophils and lymphocytes.

DIRECTED MIGRATION OF LEUKOcytes along chemical gradients is fundamental to development of lymphatic tissues (*1*), lymphocyte recirculation (*2*), and accumulation of leukocytes at sites of inflammation or tissue injury (*3*). The kinetics and the site specificity of lymphocyte infiltration, as in delayed type hypersensitivity (DTH) skin lesions (*4*), suggest that leukocyte emigration may be directed by local cellular release of cytokines. We describe the purification and characterization of an 8-kD basic heparin-binding T cell chemotactic polypeptide from the conditioned media of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) (*5*). This T cell chemotactic factor (TCF) appears to be biologically and biochemically identical to a neutrophil-chemotactic factor that is also a neutrophil-activating protein (NAP-1) that we purified and molecularly cloned (*6, 7*).

TCF was purified by sequential chromatography on heparin Sepharose (*8*), carboxymethylation exchange high-performance liquid chromatography (CM-HPLC) (*9*), and reversed-phase HPLC (*10*). To assess T cell chemotactic activity, we adapted a Boyden chamber assay (*11, 12*). The CM-HPLC fractions (fractions 52 and 53) with lymphocyte chemoattractant activity at 0.4*M* NaCl were pooled and applied to a reversed-phase HPLC column with high activity recovered in fractions 47 and 48, corresponding to a major absorbance peak that eluted at 47 min (Fig. 1). We confirmed the purity of TCF (fraction 47) by SDS–polyacrylamide gel electrophoresis (PAGE) analysis (*13*). Under reducing conditions, a single band of about 8 kD was detected. We consistently ($n = 3$) obtained pure TCF, with a yield corresponding to about 10 μg of TCF per liter of conditioned

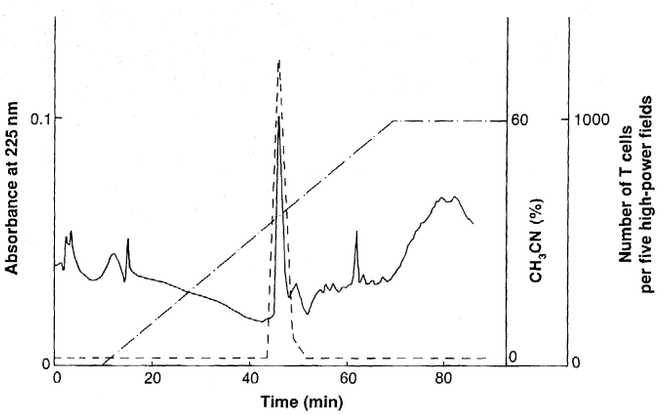

Fig. 1. T cell chemotactic activity purified from conditioned media of PHA-stimulated human PBMC by reversed-phase HPLC (*9, 12*). Absorbance is shown by the solid line; chemotactic activity, dashed line; and acetonitrile gradient, dashed and dotted line.

C. G. Larsen, J. J. Oppenheim, K. Matsushima, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick, MD 21701.
A. O. Anderson, Laboratory of Respiratory and Mucosal Immunity, Disease Assessment Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701.
E. Appella, Laboratory of Cell Biology, National Cancer Institute, Bethesda, MD 20892.

*To whom correspondence should be addressed.