

Cytodestructive Mechanisms Provoked by Food Antigens

I. Direct, Allergic Autocytotoxicity*

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In vitro challenge of peripheral white blood cells (WBC) from 62 food sensitive patients and 22 asymptomatic control individuals was performed using standardized extract of cow milk, corn and wheat. 81% of the patients reacted at least toward one food antigen, showing disintegration of WBC, as measured by trypan blue exclusion technique. The presented experimental condition of the direct binding between food antigen and cell wall membrane suggests that this phenomenon is multicellular in its origin. The interpretation of these studies is of distinct value in directing human food hypersensitivity research in the future.

Key words: allergic autocytotoxicity; food antigens.

Accepted for publication 8 October 1984

While there is still considerable disagreement concerning the role of hypersensitivity reactions induced by ingestants, food allergy is widely perceived as a major health problem (2, 4, 5, 7, 10, 19, 21, 33).

It is paradoxical that the progeny of our species might be compromised by altered processing of vital nutrients (exogenous antigens), assuming that the most important function of the immune system is the antropocentric preservation of *homo sapiens*.

Cellular death in the immune response has been investigated according to the killing potential measured by effector and target cell interaction (24). It is surprising how little attention is paid to the logical sequence of anaphylactic injury: degranulation, autocytolysis and disintegration.

Identification of autocytotoxic cells capable

of lysing syngeneic cells in animals and man essentially supports the concept of autocytolysis (23, 39). An experimental study of tolerance induced by the oral administration of antigen revealed time- and dose-dependent variances in enhancement and suppression of the immune response (30). Therefore, the aberrant functional deletion of the autocytotoxic cells (clonal anergy) might be associated with a variable stimulus-response signal delivered by ingested food antigen in susceptible individuals.

The test model system presented here is simplified and based on a restricted assumption that at least a fraction of ingested food antigen circulates in the patient's bloodstream (9, 18, 29). Incurred antigenemia is confronted by nearly all circulating white blood cells (WBC), thereby exposing susceptible hosts to sensitization (7).

In our study, WBC from food-sensitive patients were activated with extracts of cow milk, corn and wheat. This assay introduced the concept of *in vitro* food challenge and was referred

* These results were presented in part at the 40th Annual Congress of the American College of Allergists, April 7-11, 1984, San Francisco.

to as allergic autocytotoxicity (ACT), since it was observed only among sensitive individuals whose autologous, circulating WBC were damaged (26).

The experimental conditions revealed three major mechanisms of ACT response. Type I, direct ACT, was observed when food antigens act directly on the membrane of target WBC. Type II mechanisms occur via a reaction termed antibody-dependent ACT. In this system, autocytolysis is induced following a bridging between the WBC and surface receptors for Fc fragment of cytophilic antibody which binds to antigen in the vicinity. Type III mechanisms are associated with spontaneous ACT. Apparently unprimed WBC disintegrate spontaneously in the absence of antigen and antibody.

This paper presents the data related to Type I, direct ACT response.

MATERIAL AND METHODS

Clinical material

Studies were conducted in 62 patients with a recurrent history of food hypersensitivity manifested mainly in the respiratory tract (shortness of breath, wheezing), ear-nose-throat (nasal discharge, congestion, postnasal drip) and the gastrointestinal tract (abdominal cramps, diarrhea). This group was compared with 22 asymptomatic control individuals. The age of the patients varied from 12 to 68 years and the age of control individuals from 16 to 53 years. These patients had a clinically confirmed tendency toward symptom exacerbations induced by the ingestion of cow milk, corn and wheat over a period of 6 months to 5 years prior to the performance of the direct ACT assay.

Separation of white blood cells

Every subject was informed of the study's purpose, and after signed consent was obtained, 5 ml of blood was collected by venipuncture into collection tubes containing EDTA as an anti-

coagulant. Essentially the method of Henson with minor modifications was used for separation of white blood cells (16). Blood from patients and controls was then dispensed into 15 ml polystyrene tubes (Fisher # 05-527-49; 15 ml graduated conical centrifuge tubes). Then 1.5 ml of 6% dextran (MW 500,000; Sigma Cta #D-5251) in physiological saline was added and the final blood volume was adjusted to 12 ml, using physiological saline. The cell suspension was gently shaken and left for sedimentation for 30 min at room temperature. Then the supernatant was collected and centrifuged at 1200 rpm for 5 min. After the removal of the supernatant, the cells were resuspended in 5 ml of 10% hemolyzate reagent (Gelman Science, Inc., Cat #51912) in physiological saline and centrifuged at 1200 rpm for 5 min. Following the lysis of erythrocytes, the remaining white blood cells were washed twice in modified Hanks' solution (MH solution, Gibco, Cat #310-4065) and adjusted to a concentration of 1×10^6 /ml. The final differential showed 73% polymorphonuclears and 27% mononuclears.

Food antigen characteristics

Standardized extracts of whole cow milk, whole corn, and whole wheat were specially prepared for this study by Hollister-Stier (Spokane, Washington). The extraction period was 18 h at 4°C for the milk, while corn and wheat were extracted for 72 h at 4°C. After clarification through 0.2 micron membranes, the extracts were lyophilized from 1 ml volume at concentrations of 1×10^{-1} weight per volume (w/v) in distilled water. PNU had the following concentration: milk - 57,000 PNU, corn - 27,000 PNU, and wheat - 10,000 PNU, measured according to the Kjeldahl method of nitrogen determination (protein nitrogen unit). All tested antigen dilutions were in isotonic MH solution prepared on the day of testing.

Direct ACT assay

Essentially two variants of direct ACT were analyzed. In the first one, a simple and conservative approach was applied using a hema-

cytometer. This was to assure the same volume in order to measure the total number of cells in the incubation mixture before and after activation with food antigen. This was followed by measurement of the percentage of trypan blue (TB) stained, non-viable cells in the hemacytometer chamber. After establishing the kinetics of disintegration of WBC, a microcytotoxic assay was developed in which only the percentage of TB-positive cells was quantitated.

Methodology of direct ACT; hemacytometer assay

After separation of WBC, the cells of patients and controls were adjusted to a concentration of $1 \times 10^6/\text{ml}$. Then 50 μl of the cell suspension was placed in the culture tubes (Fisher #14-956-3A, 12 $\times$ 75 mm) and 50 μl of food antigens, dissolved in MH in a concentration of 1×10^{-1} to 1×10^{-6} w/v was added. In the control tubes, instead of antigen, 50 μl of MH solution was added. The total number of cells was counted in the hemacytometer. Then the culture tubes were incubated in triplicate in a shaking water bath for 2 h at 37°C. Following the incubation period, 50 μl of 0.1% TB solution in distilled water was added to each sample tube. The total number of cells and non-viable TB-stained cells were counted in a hemacytometer.

Methodology of direct ACT; microplate microassay

Sterile polystyrene microplates with flat bottom micro wells (Corning #25860) were used for the assay. Separated white blood cells from patients and controls were adjusted to a concentration of 2×10^6 cells/ml. Cow milk, wheat and corn lyophilized antigens were resuspended in 1 ml of MH and further dilutions were made in concentrations of 10^{-1} and 10^{-4} w/v. 25 μl WBC cell suspension and 25 μl of antigen solution were mixed in one well in triplicate. Proper controls were established. Microplates were gently shaken and incubated at 37°C for 2 h in a water bath. After incubation to each well, 25 μl of 0.1% TB solution in distilled water was added. The percentage of non-viable TB-stained cells was counted directly on the invertoscope (Olympus, Model IMT).

Calculation of results

The total number of cells was counted in a hemacytometer to assure equivalent cell number in the same volume before and after incubation with antigen. The percentage of non-viable cells was expressed as the specific autocytotoxic index (SAI) which was calculated from the formula:

$$\% \text{ Specific Autocytotoxic Index (SAI):} \\ \frac{\text{Exp. \% Tr. Blue with Antigen} - \text{Diluent \% Tr. Blue}}{\text{Maximal \% Tr. Blue} - \text{Diluent \% Tr. Blue}} \times 100$$

The experimental percentage of TB positive cells was determined separately for patients and controls. The maximal percentage of TB for each patient and control was calculated in each experiment using distilled water instead of an antigen solution. This value was variable from 55% to 70%. Spontaneous cytotoxicity reflected the percentage of TB-positive cells incubated without antigen and/or antibody and was variable in this group from 2–6%. SAI cytotoxic index was called specific, since its relative value was adjusted to the percentage of spontaneous and maximal disintegration of WBC in this test cell system.

Electron microscopy studies

Direct activation of WBC from a patient with cow milk allergy, manifested as bronchial asthma, and a control's WBC were analyzed *in vitro* with an extract of cow milk and incubated in a water bath for 1 h at 37°C. Following isolation, the cells were examined in an electron microscope. For details, see the methodology of our previous case report (28).

RESULTS

Two functional parameters of WBC disintegration were observed. The first one consisted of the total decrease of cell number after antigen activation *in vitro*. As illustrated in Fig. 1, such

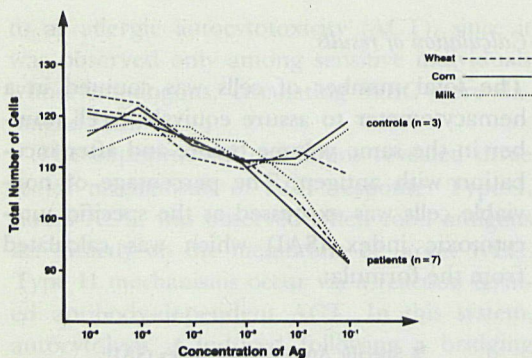


Fig. 1. Changes in total number of cells in hemacytometer chamber as a function of increasing antigen concentrations (w/v), mean values.

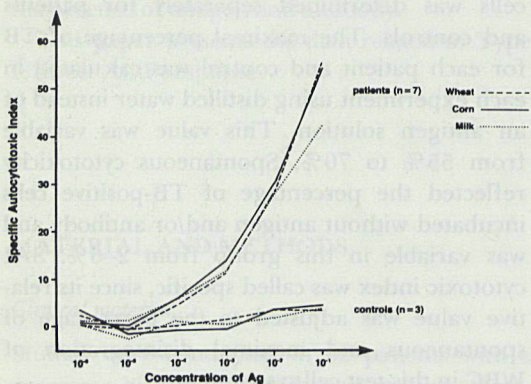


Fig. 2. Increasing direct ACT as a function of increasing antigen concentration (w/v), mean values.

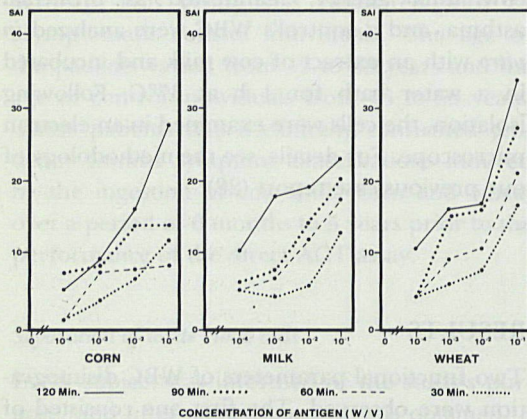


Fig. 3. Kinetics of direct ACT response. Mean value of five patients sensitive to cow milk, corn and wheat.

cellular disintegration was uniform with all three antigens and reached the maximum in the antigen concentration of 1×10^{-1} w/v. The second manifestation observed among WBC activated with food antigens was related to an increased number of non-viable TB-stained cells. This effect is summarized in Fig. 2. Increasing percentage of non-viable cells was parallel to increased concentration of the food antigen extract.

The kinetics of the ACT response related to antigen concentration and time were measured in a microplate microassay. Fig. 3 illustrates the results of the maximum autolytic capacity observed in 2 h of incubation induced by the highest concentration of antigens (1×10^{-1} w/v) in direct ACT assay. This study was conducted among five patients sensitive to corn, cow milk and wheat.

Represented cytodynamic disturbances were manifested morphologically by cellular membrane injury, enlarged cytoplasmic granules, and many intercellular junctions between mononuclears and polymorphonuclears with subsequent penetration of TB dye into the dead cells. Fig. 4 illustrates typical targeted cells with microinjection of cytotoxic content.

Limited electron microscopy studies were performed in a patient sensitive to cow milk, manifested as bronchial asthma, and WBC from one normal individual control. Monocytes from patients and a normal control individual activated with cow milk extract showed increased pinocytic-secretory profile with high plasma membrane activity and many vacuoles. Interestingly, we did not observe any basophils in our blood specimen; however, patients' eosinophils revealed a prominent discharge of granules compatible, morphologically, with release of cytotoxic major basic protein (28). Fig. 5 illustrates the typical ultrastructure of patient eosinophils and monocytes involved in direct ACT response. Electron microscopy studies did not provide evidence of morphological abnormalities among eosinophils of normal control individual.

31 patients and 13 controls were tested in the hemacytometer assay and microplate microassay. Both methods demonstrated similar re-



Fig. 4. Intercellular junctions observed among patient WBC activated with cow milk extract. Targeted cells show characteristic microinjection of cytotoxic content, see arrow.

producible response. In addition, 31 patients (total 62 patients) and nine controls (total 22 individuals) were tested in the microplate microassay only. Table 1 represents the summary of the data of direct ACT assay, using the microplate microassay. It should be emphasized that resultant damage in direct ACT at least to one food antigen occurred in 81% of patients. The observed positive reaction ($SAI > 10\%$) was antigen selective. The ACT assay is reproducible, when the same patient is tested on the next day.

DISCUSSION

Several methodological conditions should be under consideration in the interpretation of direct ACT response.

One of the earliest means used to assess nucleated cell killing was through the uptake of vital dyes such as TB, by dead cells. This technique is simple, rapid, and involves minimal manipulation of cells for assessing their viability in cellular attack test systems (32, 36). It is very important to recognize that the TB dye is not an inert molecule. Many observations suggest that TB might serve as a carrier for arginine rich polycationic proteins (1, 11, 13, 35). Poly-arginine ligands attached to the TB molecules might therefore be toxic to the cell wall membrane. Since leukocyte lysosomes are known to be rich in cationic proteins, the introduction of TB in our assay might be associated with amplification of the autolytic system response (12).

Since the cell membrane is the first barrier between the cell and its environment, one could expect that measurement of its permeability to water is a sensitive criterion of altered cellular function. Indeed, studies of membrane perme-

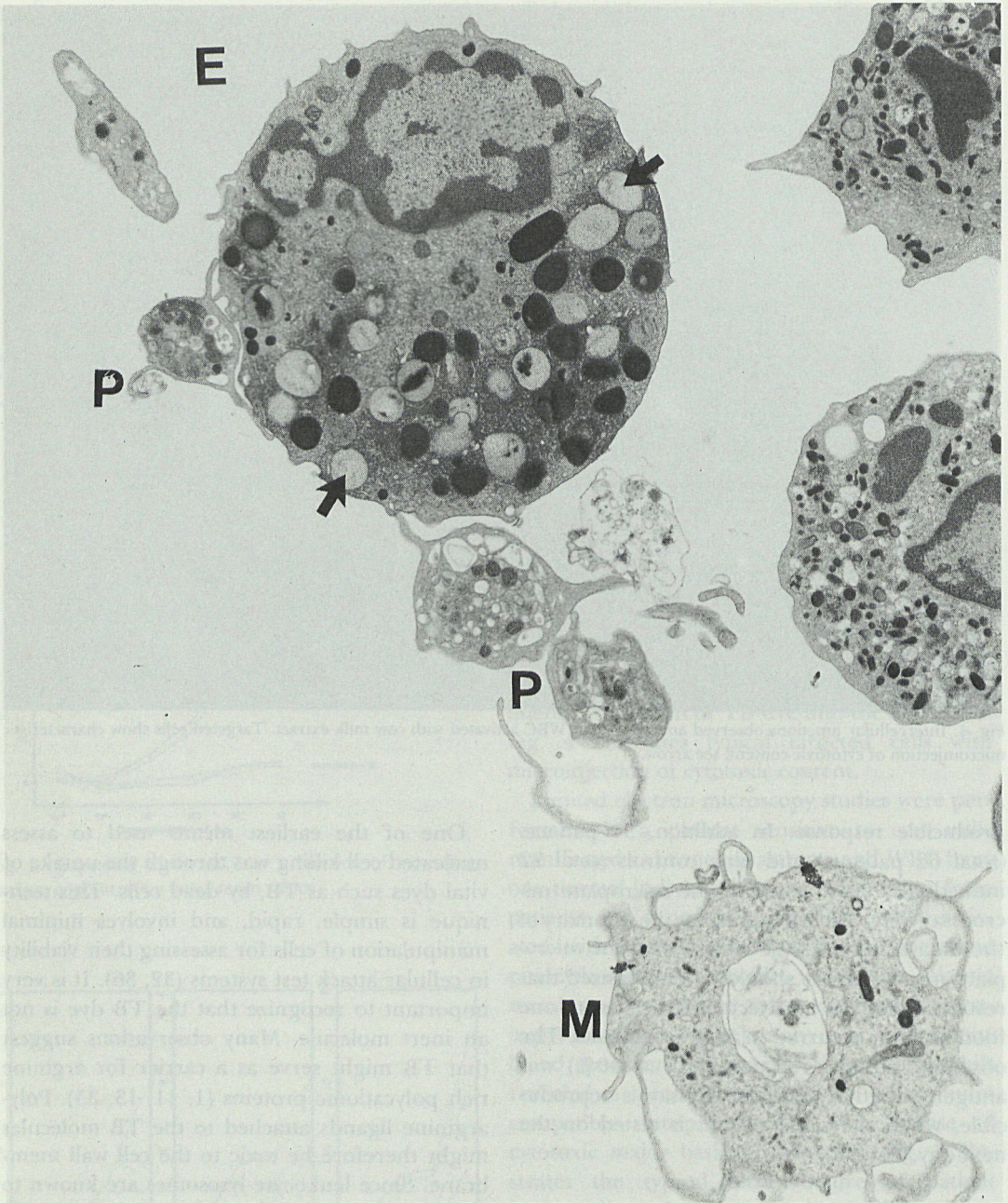


Fig. 5. Ultrastructure of patient eosinophil (E) and monocyte (M) activated with cow milk extract. Arrows indicate eosinophil degranulation discharges (magnification $\times 9100$). P = platelets.

ability to water distinguished different transportation pathways for normal and neoplastic cells (15).

Assuming that all cells function as osmo-

meters, the application of TB solution in distilled water in our test system was to trigger pre-existing cell wall membranes anomaly. Indeed, every subject had different spon-

Table 1
Summary of direct ACT response among 62 patients under study

Food antigen: Patients:	Reactive to at least one food antigen*	Reactive to cow milk*	Reactive to corn*	Reactive to wheat*
Total number of patients reacted (SAI > 10%)	50	26	19	33
% of reacted patients	81%	42%	31%	53%

Among 22 asymptomatic control individuals only 1 reacted towards milk antigen.

* Concentration of antigen 1×10^{-1} w/v.

taneous and maximal uptake of TB at the same experimental condition. There may be sufficient variances in the permeability values for each cell type and each group; asymptomatic control versus food hypersensitive patients. This fact perhaps contributes to the characteristic of our methods. Therefore, WBC of sensitive individuals may be excessively and pathologically hyperreactive to a membrane signal delivered by food antigen. The decreased cellular membrane threshold of WBC is possibly a general characteristic of cells involved in anaphylactic injury induced by food antigens. This is of particular reference to physical trauma in food-dependent, exercise-induced anaphylaxis (6, 34).

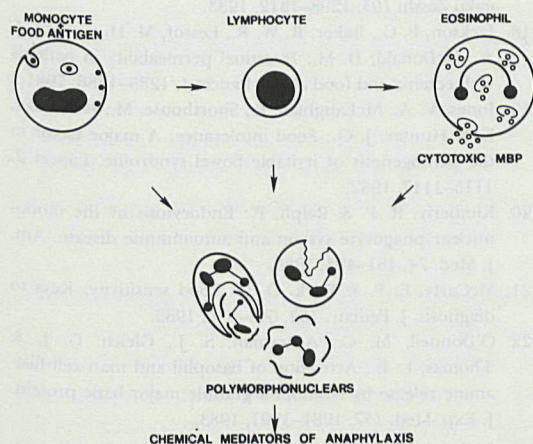


Fig. 6. Hypothetical multicellular model of direct ACT response. Eosinophil discharges represent cytotoxic major basic protein.

At the present time, neither the functional profiles of involved cells nor clinical relevance of ACT phenomenon are known. However, in most circumstances, direct antigen binding seems to be related to the recognition by the cells, and thus a high frequency of specific binding above background level was observed. Despite the fact that extracts of wheat and corn are equivalent of plant lectins, which induce non-specific lysis, the observed direct ACT response was antigen selective when the reaction toward extract of cow milk, corn and wheat were compared in the same individuals (see Table 1).

Observation of direct ACT induced by food antigens might contribute to our general understanding of why individuals with allergies have a low incidence of malignancies. Since lectins, like wheat germ agglutinins, can activate human polymorphonuclears toward lysis of tumor cells, it is plausible to suggest that food hypersensitive individuals may possess a hidden cellular killing mechanism which protects them against dissemination of malignant tumor cells (17, 37, 38).

The ACT response is a complex multicellular phenomenon in its origin. Fig. 6 illustrates hypothetical antagonism and synergism between WBC subpopulations which might culminate in ACT response. Monocyte function is focused presumably on phagocyte system clearance and determination of the antigenic signal which is of paramount importance for the initiation of immunological response (20). In addition, our limited electron microscopy study demonstrated eosinophil degranulation in direct ACT

assay. This observation supports the concept of cytotoxic major basic protein release from eosinophils, a molecule which can be either cytotoxic to bystander cells or can induce non-cytotoxic histamine release from basophils (22).

In the past, significant rises in plasma histamine levels were observed in sensitive individuals challenged with the offending food antigens (3, 31). In order to elucidate the anaphylactic background of direct ACT response, the immunomodulatory role of ketotifen, an antagonist of chemical mediators of anaphylaxis, was examined (8, 14). Our preliminary results revealed that ketotifen inhibits direct ACT response (27). Currently, the investigation of chemical mediators of anaphylaxis in ACT assay is under study in our laboratory.

Despite the fact that, in the past, autocytolysis of leukocytes after exposure to food antigens was observed, a more sophisticated and detailed scientific approach was never initiated (25). This perhaps reflects the fact that studies of the cytodynamic disturbances induced by food antigens are less attractive for scientists and are in their infancy as diagnostic or therapeutic tools for clinicians. Therefore, the use of standardized, quantitative measures in assessing ACT response may be very critical in monitoring *in vivo* oral challenge reactions provoked by food antigens.

At this time, it is impossible to prove that all other forms of food allergy may respond in the same manner.

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