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ASSESSMENT OF CELL-MEDIATED IMMUNITY IN THE RAT UTILIZING THE AGAROSE-DROPLET CELL-MIGRATION-INHIBITION CORRELATE OF DELAYED-TYPE HYPERSENSITIVITY

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SUMMARY: The agarose-droplet cell-migration-inhibition assay was developed for measuring specific cell-mediated immunity in rats. Fischer #344 female rats were sensitized to mono(p-azobenzene-arsonate)-N-chloroacetyl-L-tyrosine (ARSNAT), keyhole-limpet-hemocyanin (KLH), or a BCG cell wall preparation. The optimal conditions for assay were determined by testing varying concentrations of antigens against normal and sensitized peritoneal exudate cells induced with 5% thioglycolate medium. Specific cell-mediated immunity to each of three different antigens was detected, which correlated with skin tests observed in vivo. Our adaptation of the agarose-droplet assay should provide a useful model for studying other aspects of cell-mediated and/or tumor immunity in the rat.

Cellular immunity is an important immune mechanism in protection against various microbial diseases and certain types of tumors. Several animal species have been used as models to study cellular immunity and/or delayed hypersensitivity in various systems, with each animal species having certain advantages and disadvantages. The rat has been used extensively in immunological experiments examining the role of alveolar macrophages in infectious diseases, assessing qualitative differences in lymphocyte populations, and studies of the immune response in tumor systems. However, little work has been done on in vitro studies of cell-mediated immunity in the rat using the cell-migration-inhibition test, an accepted in vitro correlate of cell-mediated immunity (1, 2, 3). Most of the previous in vitro cell-migration-inhibition assays with animals and humans have measured the migration of peritoneal exudate cells (PEC) from capillary tubes utilizing the direct and indirect methods, respectively. Various studies attempting to utilize the capillary-tube method in rats have generally been limited, mainly because of small yields of PEC obtained and difficulty in demonstration of immunological specificity.

In 1973, Harrington and Stastny (4) introduced an agarose-droplet cell-migration-inhibition assay micro method for measuring cell migration inhibition

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(a correlate of cell-mediated immunity) in the guinea pig and mouse (1). This technique has the advantages of being technically simple, permitting setting up a large number of assays with smaller numbers of peritoneal exudate cells from a single animal, and requiring only small amounts of tissue culture reagents, antigens, and incubation space.

The objective of the present study was (a) to demonstrate the specific cell-migration-inhibition correlate of cellular immunity in the inbred rat, attempting to utilize the agarose-droplet cell-migration-inhibition method; (b) to use this assay to detect cellular immunity against a chemically defined antigen, mono(p-azobenzene-arsonate)-N-chloroacetyl-L-tyrosine (ARSNAT), a non-chemically defined antigen, keyhole-limpet-hemocyanin (KLH), or a BCG cell wall preparation; and (c) to compare the *in vitro* cell-migration-inhibition data with the results of *in vivo* skin testing.

MATERIALS AND METHODS

Animals. Four to five week old Fischer #344 female rats were obtained from Charles Rivers, Wilmington, Mass. The animals were housed individually and fed daily a diet of standard rat chow and water *ad libitum*.

Antigens. The preparation of the mono(p-azobenzene-arsonate)-N-chloroacetyl-L-tyrosine (ARSNAT) has been described by Tabachnick and Sobotka (5) and by Leskowitz *et al*. (6). Arsanilic acid is diazotized and coupled to the N-acyl derivative of L-tyrosine (Sigma Chemical Co., St. Louis, Mo.). A 50 ml solution of .06M diazotized arsanilic acid was prepared by the addition of 3 mmol of arsanilic acid (Eastman Organic Chemicals, Rochester, NY) to 0.5N HCl using 0.6 mmol of sodium bromide as a catalyst. After diazotizing for 1 hr, the arsanilic acid solution was slowly added with constant stirring to 3 mmol of N-chloroacetyl-L-tyrosine in 25 ml of 0.01M borate buffer (pH 9.3) at 0°C. After 4 hr the mixture was acidified by adding 0.5N HCl until the pH was 1.8. The resulting orange precipitate was collected and washed six times with cold distilled water acidified with 1N HCl to a pH of 1.8. The ARSNAT antigen was redissolved in 1N NaOH (pH 9.0) and reprecipitated three times by alternate elevation of the pH to 9.0, followed by lowering the pH to 1.8 with 0.5N HCl. The final product was dried and stored in the absence of light in a cool, dry atmosphere.

Keyhole-limpet-hemocyanin (KLH) was purchased in lyophilized form from Cal Biochem, La Jolla, Ca. BCG cell wall preparation Lot #179 was kindly supplied by Dr. Edgar Ribi, Rocky Mountain Laboratory, NIAID, Hamilton, Mt. 59840. PPD was obtained from Control Veterinary Laboratory, Surrey, England. Coccidioidin was purchased from Cutter Laboratories, Inc., Berkeley, Ca., and diluted 1:10,000 in Hank's balanced salt solution for use in tissue culture.

Sensitization. Rats were sensitized against ARSNAT by footpad injection of 150 µg of ARSNAT dissolved in 0.5 ml of sterile, normal saline and emulsified in 0.5 ml of Freund's complete adjuvant (FCA) containing 500 µg/ml of *Mycobacterium butyricum* (Difco Laboratories, Detroit, Mi.). Rats were sensitized to KLH by injection into the footpads of 250 µg of KLH dissolved in 0.5 ml of Hank's balanced salt solution (HBSS) and emulsified in an equal volume of FCA. Rats were

sensitized against BCG by intravenous (IV) tail vein injection of 150 µg of BCG cell wall admixed with 2 µl of Drakeol 6-VR (Pennsylvania Refining Co., Butler, Pa.) and emulsified in 0.2 ml of 0.2% Tween-80-saline solution, as previously described by Ribi et al. (12-13).

Skin testing. Two to four weeks after immunization, the rats were skin tested in the left ear with 0.1 ml of sterile, normal saline containing either 10 µg of ARSNAT, 20 µg of KLH, or 10 µg of purified protein derivative (PPD). All right ears were injected with 0.1 ml of sterile, normal saline. Injections were done with tuberculin syringes and 28 gauge needles. Skin tests were observed at 2, 18, 24 and 48 hr after injection of each antigen and saline control. Rat ear thickness was measured at 2, 24 and 48 hr with a micrometer. A skin test was considered positive if the ratio (R) was greater than 5:1 where R = A•D/B•C and:

A = 24 hr reading (mm) of antigen-injected ear
B = 0 hr reading (mm) of antigen-injected ear
C = 24 hr reading (mm) of saline-injected ear
D = 0 hr reading (mm) of saline-injected ear

Collection of peritoneal exudate cells. Rat peritoneal exudate cells (RPEC) were obtained from animals injected intraperitoneally (IP) three days earlier with 25-30 ml of 5% sterile thioglycolate medium (Difco Laboratories, Detroit, Mi.) dissolved in sterile, 85% saline. The RPEC were collected by multiple washes of the peritoneal cavity with HBSS and pelleted by centrifugation at 500 x g. The cells were pooled, washed three times with HBSS, and divided equally into two sterile, graduated 15 ml glass conical centrifuge tubes.

Agarose-droplet cell-migration-inhibition assay. The assay for cell-migration-inhibition is similar to the procedure of Harrington et al. (4) and has been described earlier (4, 5). During the final wash with HBSS, each pellet of RPEC is centrifuged in the graduated glass conical centrifuge tubes at 500 x g and resuspended in a 1:4 ratio of cells to tissue culture medium 199 (Difco Laboratories, Detroit, Mi.) containing 30% heat-inactivated (HI) normal rat serum and 10% HI fetal calf serum in an equal volume of 0.4% sterile agarose (Marine Colloids, Rockland, Ma.). The RPEC were drawn into a sterile, 50 µl Hamilton syringe fitted with a pushbutton dispenser. A volume of 1 µl of agarose-cell suspension (5 x 10^5 to 1 x 10^6 RPEC) was deposited on the center surface of each well of a flat-bottomed sterile micro-titer plate (Bellco Glass, Vineland, NJ), and the droplets were permitted to solidify at 5°C in the refrigerator for 8 min. The plates were removed from the cold and 0.25 ml of RPMI 1640 media containing 10% HI normal rat serum and 5% HI fetal calf serum and the antigens were carefully added to each well. After 48 hr, the droplets were examined under a Zeiss inverted microscope with a 2.5X objective and an ocular containing a 100 x 100 mm grid. The number of squares of cellular migration from four equidistant points at the edge of the droplet were counted and recorded.

Analysis of data. The analysis of migration data is the same method reported in earlier publications (7, 8, 9, 10). The migration of RPEC were measured from eight agarose droplets and utilized to calculate the values shown in Tables 1-4. A mean migration index (MMI) was calculated on an IBM 360 computer using the following formula, where n represents and number of measured migration distances of RPEC incubated alone, and m represents the number of measured migration areas of RPEC under different experimental conditions. Summation of the values for n and m are represented by i and j, respectively.

$$MMI = \sum_{i=1}^{n} \sum_{j=1}^{m} \frac{\text{Area of migration of RPEC under experimental conditions } j}{\text{Area of migration of RPEC incubated alone } i} \times 100$$

$$\frac{}{n \times m}$$

TABLE 1

Mean Migration Indices of Normal RPEC Incubated with ARSNAT, Keyhole-Limpet-Hemocyanin (KLH), Purified Protein Derivative (PPD), or Coccidioidin Antigens

ANTIGEN	ARSNAT (μg)			KLH (μg)				PPD (μg)	COCCIDIOIDIN
Animal	10[b]	20	50	50	100	200	400	20	1:10,000
1[a]	98±3	97±3	88±3	89±2	91±2	88±2	85±3	93±2	94±4
2	92±1	87±3	86±4	94±4	92±4	90±3	94±3	89±1	90±2
3	94±2	95±4	88±1	85±2	91±4	89±4	96±3	92±2	87±3

[a]Ear swelling ratios were 1.0 for animals 1, 2, and 3.
[b]Number of micrograms of antigen per ml.

RESULTS

Unimmunized controls. Table 1 shows the results of MMI's of unimmunized, "nonsensitized" control RPEC incubated with each of the antigens at varying concentrations. ARSNAT, KLH, PPD, or coccidioidin failed to inhibit the migration of RPEC; MMI's ranged from 86±4 to 98±3 for ARSNAT, 85±2 to 96±3 for KLH, 89±1 to 93±2 for PPD, and 87±3 to 94±4 for coccidioidin.

ARSNAT-sensitized RPEC. Sensitized RPEC from rats immunized against ARSNAT were incubated in the agarose-droplet assay with ARSNAT antigen, KLH, PPD, or coccidioidin (Table 2). The nonspecific antigens, KLH and coccidioidin, failed to inhibit the migration of RPEC; MMI's ranged from 94±4 to 102±5 and 90±4 to 108±4, respectively. On the other hand, the ARSNAT-sensitized RPEC were inhibited by specific antigen at 3 separate concentrations; MMI's ranged from 29±3 to 69±4. ARSNAT-sensitized RPEC were also inhibited by PPD; MMI's ranged from 31±2 to 38±4. Because the animals were immunized against the ARSNAT antigen emulsified in Freund's complete adjuvant, cell-mediated hypersensitivity to PPD would be expected and represents an internal positive control in these experiments.

TABLE 2

Mean Migration Indices of ARSNAT-Sensitized RPEC Incubated with ARSNAT, KLH, PPD, or Coccidioidin Antigens

ANTIGEN	ARSNAT (μg)			KLH (μg)	PPD (μg)	COCCIDIOIDIN
Animal	10	20	50	200	20	1:10,000
1[a]	69±4	57±3	39±4	102±5	38±4	108±4
2	64±3	49±1	29±3	96±2	31±4	90±4
3	60±3	47±2	32±4	94±4	31±2	91±3

[a]Ear swelling ratios were 6.9, 5.0, and 6.3 for animals 1, 2, and 3, respectively.

KLH-sensitized RPEC. Sensitized RPEC from animals immunized with KLH were incubated with the specific antigen (KLH), ARSNAT, PPD, or coccidioidin (Table 3). Again, ARSNAT or coccidioidin failed to inhibit the migration of RPEC; MMI's ranged from 97±3 to 100±5 for ARSNAT and 95±5 to 103±4 for coccidioidin. In contrast, the KLH-sensitized RPEC were inhibited by the specific antigen, KLH, at all concentrations; MMI's ranged from 29±4 to 66±5. Similarly, as was seen with the ARSNAT-sensitized RPEC, KLH-sensitized RPEC were also inhibited by PPD; MMI's ranged from 29±4 to 30±2.

BCG-sensitized RPEC. Sensitized RPEC from rats immunized with a BCG cell wall preparation were incubated with PPD, ARSNAT, KLH, or coccidioidin (Table 4). ARSNAT, KLH, or coccidioidin failed to inhibit the migration of RPEC; MMI's ranged from 85±2 to 89±3 for ARSNAT, 90±3 to 98±2 for KLH, and 94±3 to 100±2 for coccidioidin. On the other hand, the BCG-sensitized RPEC were inhibited by the specific antigen, PPD, at all concentrations; MMI's ranged from 15±5 to 67±4.

DISCUSSION

With the use of the agarose-droplet cell-migration-inhibition test, we were able to detect specific cell-mediated immunity in Fischer rats immunized with

TABLE 3

Mean Migration Indices of KLH-Sensitized RPEC Incubated with Specific Antugen (KLH), ARSNAT, PPD, or Coccidioidin Antigens

ANTIGEN	KLH (μg)				ARSNAT (μg)	PPD (μg)	COCCIDIOIDIN
Animal	50	100	200	400	50	20	1:10,000
1[a]	64±4	57±3	37±4	33±4	98±5	30±2	100±5
2	61±3	43±3	33±3	29±4	97±3	33±4	103±4
3	66±5	59±5	36±4	29±4	100±5	29±4	95±5

[a]Ear swelling ratios were 6.9, 5.9, and 6.1 for animals 1, 2, and 3, respectively.

either the chemically defined antigen, ARSNAT, the non-chemically defined protein antigen, KLH, or a BCG cell wall preparation. The results of the cell-mediated reactivity to specific antigen in the cell-migration-inhibition test correlated with the *in vivo* skin tests in the rats. *In vitro*, the migration of RPEC sensitized to ARSNAT, KLH, or BCG was markedly and specifically inhibited by the sensitizing antigen; these same antigens failed to inhibit the migration of RPEC from unimmunized animals or animals immunized against nonspecific antigens. Because the cell-migration-inhibition assay represents a qualitative, and not quantitative, assessment of delayed hypersensitivity, our laboratory has adopted a conservative value for a result representing positive inhibition. This value for positive inhibition is a MMI of 65% or below and is based on a statistical analysis of the assay performed by Bergstrand and Kallen (11). Other laboratories have experienced difficulty in obtaining sufficient numbers of PEC in the rat utilizing peptone solutions, starch, light mineral oil, and dextran for performing similar studies. We have not had this problem utilizing the thioglycolate-induced peritoneal exudates.

Rat models have advantages over other animal models for certain immunologic studies. Rats are of adequate body size and are susceptible to microbial pulmo-

TABLE 4

Mean Migration Indices of BCG-Sensitized RPEC Incubated with Specific Antigen (PPD), ARSNAT, KLH, or Coccidioidin Antigens

ANTIGEN	PPD (μg)			ARSNAT (μg)	KLH (μg)	COCCIDIOIDIN
Animal	10	20	40	50	200	1:10,000
1[a]	56±4	35±5	15±5	89±3	93±2	100±2
2	57±4	39±4	26±5	85±2	90±3	94±3
3	67±4	56±3	29±4	89±1	98±2	96±3

[a]Ear swelling ratios were 6.0, 5.9, and 6.0 for animals 1, 2, and 3, respectively.

nary disorders as well as virally and chemically induced tumors. This adaptation of the agarose-droplet cell-migration-inhibition test in assessing specific cell-mediated immunity to antigens provides an economical and technically simple tool for *in vitro* studies of cell-mediated immunity in the rat in these disease models.

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