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In Vivo and In Vitro Cellular Responses to Cytoplasmic and Cell Wall Antigens of *Histoplasma capsulatum* in Artificially Immunized or Infected Guinea Pigs

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Guinea pigs were infected with different doses of yeasts of *Histoplasma capsulatum* or artificially immunized with several concentrations of unextracted yeast cell walls, and then tested in vivo and in vitro for cell-mediated responses to various subcellular fractions of the fungus. Three types of cell-mediated responses were measured, viz., skin test activity, production of migration inhibition factor, and lymphocyte transformation. Positive cutaneous reactions were elicited in animals immunized with 100 or 1,000 μg of cell walls when such animals were skin-tested with cell wall glycoprotein or soluble cytoplasmic substances, whereas animals immunized with 2,000 μg of cell walls did not react significantly greater than unsensitized animals when skin-tested with the same antigens. Histoplasmin did not elicit cutaneous sensitivity in guinea pigs infected with the smallest inoculum, 6×10^5 yeast cells, or in animals immunized with cell walls, regardless of the concentration of cell walls used as immunogen. However, hypersensitivity to *H. capsulatum* could be detected with cytoplasmic substances in animals infected with 6×10^5. In guinea pigs infected with larger doses, i.e., 10×10^7, 15×10^7, or 20×10^7, hypersensitivity could be detected with histoplasmin, cell wall glycoprotein, a ribosome-rich fraction, and soluble cytoplasmic substances. Both cell wall glycoprotein and soluble cytoplasmic substances were functional in migration inhibition factor assays with peritoneal exudate cells from animals immunized with 100 or 1,000 μg of cell walls. The transformation of lymphocyes from infected and artificially immunized guinea pigs in the presence of cell wall glycoprotein and soluble cytoplasmic substances was variable and unpredictable, the lymphocytes from some animals within a given group transforming and those from other animals showing no evidence of stimulation. Moreover, the level of stimulation could not be correlated with the degree of dermal hypersensitivity. These findings suggest that cell wall glycoprotein, and the fractions containing ribosomes and soluble cytoplasmic substances, could be useful antigens in assays for cellular immunity, and warrant further investigation with respect to specificity and active components.

Protective immunity to *Histoplasma capsulatum* is thought to be of a cellular, rather than humoral, type (3, 12), and at least some of the antigens responsible for eliciting the protective response are believed to be in the cell wall (9, 18). Moreover, a positive cutaneous reaction to histoplasmin (HP), a culture filtrate derived from growth of the mycelial phase in a liquid medium, is a cellular response, and recovery from infection with retention of positive reactivity is generally believed to be indicative of resistance to reinfection. Although some attempts have been made to isolate the reactive components of HP (e.g., 11, 20, 21), there remain many problems associated with the use of culture filtrate antigens, and the precise fractions of *H. capsulatum* responsible for the stimulation of protection and elicitation of positive cutaneous reactivity have not been demonstrated (13, for a review).

We are attempting to attack the problem of cellular immunity in histoplasmosis by using the organism itself, rather than a product of its growth, and have been focusing attention on the cell walls because of their implication in protection. However, because of the inherent difficulties of ascertaining the purity of preparations of cell walls, cytoplasmic constituents have been included in the assay systems employed. The purpose of this portion of the inves-

tigation, then, was to attempt to demonstrate a cell-mediated response in guinea pigs immunized with cell walls, and to compare responses in artificially immunized animals with those sensitized by infection. Only the yeast phase of *H. capsulatum* was used, as it is the tissue form, and the immunological response must be to some component of it.

MATERIALS AND METHODS

Cultural methods and fractionation procedures. *H. capsulatum* SwA, employed throughout these studies, was maintained in the yeast phase on brain heart infusion agar at 37 C by weekly transfer. *H. capsulatum* SwA is a representative of chemotype I, a typing system based on the chemical nature of the cell wall (5).

A flow diagram illustrating the preparation of the subcellular fractions used for immunization, skin testing, or in the in vitro tests for cellular immunity is presented in Fig. 1. Yeast cells were obtained in large quantities by incubation in soy dialysate broth (17) for 72 h at 37 C in a gyrotory shaker with constant aeration. They were harvested by centrifugation, washed three times with tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.2) containing 0.01 M $MgCl_2$, and stored at −20 C suspended in the same buffer. Precise details for homogenization of the yeast cells, and for the lipid and ethylene-diamine extraction of cell walls, have been described elsewhere (6). The only deviation from the procedures previously described was the use of the Tris buffer as stated above. Since one of the subcellular fractions to be used as a test antigen was expected to be rich in ribosomes, a Tris buffer was used to better maintain them in their native configuration. After isolation, the cell wall glycoprotein (GP) and the soluble cytoplasmic substances (SCS) were dialyzed against distilled water and stored in the lyophilized state. The ribosome-rich pellet (RR) was resuspended in the Tris-$MgCl_2$ buffer and stored at −20 C in 1-ml aliquots. The protein content of GP, RR, and SCS was determined by the Lowry method (14), and the ribonucleic acid content of RR and SCS was measured by the orcinol method (19).

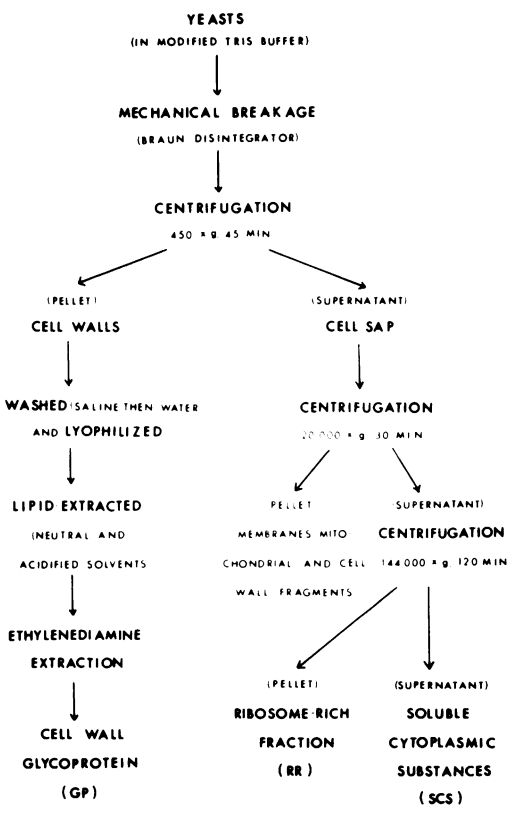

FIG. 1. *Schematic representation of the steps taken to prepare subcellular fractions of yeast cells of H. capsulatum.*

Artificial immunization. Hartley male guinea pigs, weighing approximately 400 g, were employed throughout. Immunization was accomplished by emulsifying cell walls, which had been homogenized in saline in a glass tissue grinder, with Freund complete adjuvant (FCA, Difco) such that the desired concentration of antigen was contained in 0.4 ml. Individual guinea pigs received 0.1 ml of the antigen-adjuvant mixture in each hind footpad and 0.2 ml subcutaneously between the shoulders. Saline control animals received saline emulsified in FCA and will be referred to as unimmunized animals.

Infection. Viable yeasts for infection were prepared by incubating *H. capsulatum* in a nonantigenic soy dialysate broth (17) for 3 days at 37 C on a gyrotory shaker at 165 gyrations per min. Preliminary experiments had shown that yeasts incubated in this manner in the stated medium were in the logarithmic phase of growth at the time of harvest. The yeasts were harvested by centrifugation and washed, the number of cells was determined by counting in a hemacytometer, and the final suspension was adjusted to the desired concentration in nonpyrogenic saline. The inoculum was always spread on Sabouraud agar plates before and after infecting the animals, to verify the viability of the inoculum. Animals were infected by the intraperitoneal (i.p.) inoculation of 5 ml of the yeast suspension or by a combination of i.p. and subcutaneous (s.c.) inoculation, 5 ml i.p. and 0.5 ml s.c.

Skin testing. Guinea pigs were skin-tested on the shaved and depilated flank by intradermal inoculation of 0.1 ml of antigen suspended or dissolved in nonpyrogenic saline, except that the RR fraction was retained in the Tris-$MgCl_2$ buffer in which it was stored. All reactions were read 4, 7, 24, and 48 h after deposition of antigen. Two features of each test were measured: increase in skin thickness and diameter of induration. Skin thickness was determined with a Schnelltaster caliper, and the readings on the dial were divided by 2 to obtain single-skin thickness. The formula $V = 4/3\ \pi r^2 t$, where r is the radius of induration and t is the thickness of induration, was used to calculate the volumes of the skin test responses (4). Bryceson et al. (4) found the vol-

ume to be more closely related to dose of antigen than either the diameter or thickness alone. Each guinea pig received multiple skin tests at one time, but on only one flank; the other flank was used for skin testing a second time. Except for HP, the antigen concentrations used in skin testing were determined by prior titration. HP was provided by Howard Larsh (University of Oklahoma, Norman) at a dilution of 1:25, suitable for skin testing sensitive guinea pigs. Its protein content was determined by the Lowry method (14). Normal guinea pigs were always injected with all skin test preparations to determine any nonspecific inflammatory capacity of the antigens. Skin test reactions were arbitrarily considered weakly positive if the volume was maximum at 24 h and if it ranged between 10 and 25 mm^3, because such volumes represented reactions with diameters of induration between 5 and 10 mm. Any reactions greater than 25 mm^3 were considered substantially positive reactions. Control reactions, with one exception, were always considerably smaller than 10 mm^3. SCS seemed to be highly immunogenic, and occasionally control animals developed weakly positive reactions to it after a single skin-test.

Migration inhibition factor (MIF) assays. Peritoneal exudate was induced in immunized animals and in unimmunized animals that had not received saline in FCA, and that had not been skin-tested, by the i.p. injection of 20 ml of sterile light mineral oil (Bayol SS, Exxon) 72 h before harvest. The unimmunized animals were included in the protocol each time to ascertain that the antigenic preparations were not of themselves causing the inhibition. Exudates from two animals per group were pooled for the assays. Each exudate was washed from the cavity with 100 ml of Hanks balanced salt solution (Difco) containing 200 U each of penicillin and streptomycin (Grand Island Biological). After washing and counting, the exudate cells were suspended to a concentration of 2×10^7/ml in RPMI 1640 without glutamine (Flow Laboratories) but containing antibiotics and 15% normal guinea pig serum, which had been heat-inactivated. Capillary tubes (blood clotting, Difco) were filled by capillary action, plugged with Sealease (Clay-Adams, Inc.) and centrifuged. Each tube was broken at the cell-medium interface and placed horizontally in a chamber with the sealed end secured in a drop of silicone grease. The chamber was filled with medium containing either no antigen or antigen at the desired concentration, and sealed by depositing a sterile cover slip on the top. The chambers consisted simply of glass rings, 13 mm in diameter and 5 mm in height, attached to a glass slide with silicone grease, and having a total capacity of approximately 1 ml. Only one capillary tube was placed in a chamber, and each antigen dilution or control was set up in quadruplicate. The chambers were incubated for 20 to 24 h, and the areas of migration were determined with the aid of a projecting microscope and polar planimetry.

Lymphocyte transformation (LT) assays. Lymphocytes derived from peripheral blood were employed in a system similar to that described by Valentine (23). Heparinized blood was sedimented in 5% dextran. After sedimentation, the lymphocytes were collected, washed with RPMI 1640 (Flow Laboratories) containing 200 U each of penicillin and streptomycin, and counted in a hemacytometer. They were then suspended to a concentration of 10^6/ml in RPMI 1640 containing 10% normal guinea pig serum and the antibiotics. Cells from sensitized animals were always run in parallel with cells from control, unsensitized animals. Cultures, with or without antigen, were incubated for 72 h in an atmosphere of 5% CO_2-95% air and were then pulsed for 24 h with [^{3}H]thymidine, 5 μCi per ml (New England Nuclear). After the pulse, they were washed and lysed, the protein was precipitated with trichloroacetic acid, and the precipitate was solubilized in Soluene (Packard). The resultant solutions were counted in a Beckman scintillation counter. Picamoles per 10^8 cells per hour were calculated, and the experimental values were divided by the control values to obtain the experimental-to-control (E/C) ratios. All cultures were dispensed in triplicate, and, if the counts per minute from two of the three tubes were not within the same range, the data from that set were discarded. E/C ratios of >3 were considered significant.

RESULTS

Chemical determinations. The GP fraction of the cell wall contained approximately 0.09 mg of protein per mg, the RR fraction of the cytoplasm contained about 2 mg of protein per ml, and the SCS component contained about 0.8 mg per mg. Therefore, animals given skin tests of 50 μg of GP actually received approximately 4.5 μg of protein, whereas those given 50 μg of SCS got about 40 μg of protein. Since a 1:10 dilution of the RR preparation was used for skin testing, a skin test dose of that fraction contained approximately 20 μg of protein. The protein concentration of the 1:25 dilution of HP was 34.3 mg/ml, and since each guinea pig received 0.1 ml of that suspension, each skin test contained 3.4 mg of protein. The RR and SCS fractions contained 60 and 5% ribonucleic acid, respectively.

Skin test reactions in artificially immunized guinea pigs. All skin test reactions referred to herein as positive were maximum at 24 h. Therefore, many of the skin test data are reported only as the 24-h reaction. However, in Fig. 2, to be discussed later, the data obtained over the entire observation period are presented to illustrate the typical patterns of the reactions observed in response to the various test preparations. Moreover, inasmuch as volume of reaction as a measure of sensitivity is not a commonly used procedure, some of the reactions are compared in Table 2 with the more conventional measurements of induration, thus allowing the reader to sense the magnitude of sensitivity as determined by volumes.

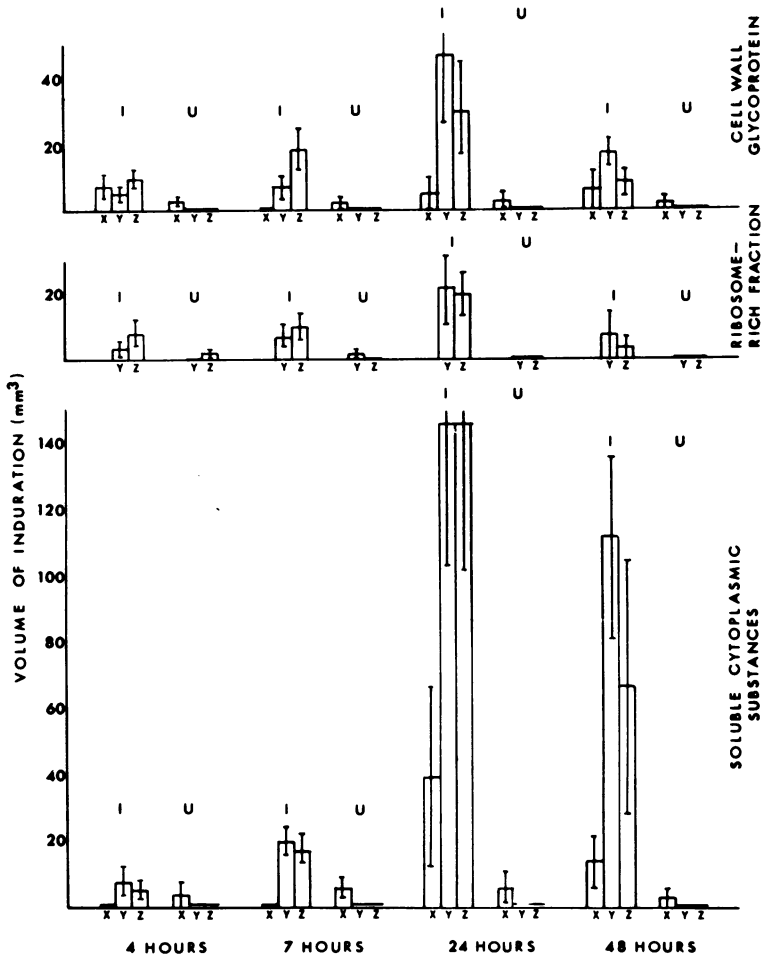

FIG. 2. *Collation of skin test data from three experiments in which guinea pigs were tested with two or three subcellular fractions of H. capsulatum 1.5 (X), 2.0 (Y), or 2.5 (Z) weeks after immunization with 100 μg of cell walls. Each bar represents the mean with a standard error of seven animals. I, Immunized; U, unimmunized.*

Initially, groups of six guinea pigs each were immunized with 2,000 or 1,000 μg of unextracted cell walls of *H. capsulatum*. All were skin-tested 3 weeks later with GP and HP, and those immunized with 1,000 μg of cell walls were skin-tested with crude cell sap as well. After those two experiments, additional guinea pigs were immunized with 100 μg of cell walls and skin-tested 3 weeks later with GP, HP and SCS instead of crude cell sap. The skin test data from these three experiments are summarized in Table 1. It can be seen from these data that guinea pigs sensitized with 100 μg of cell walls were more reactive to both GP and cytoplasmic substances than those animals receiving the larger doses. A direct comparison of the reactions to the cytoplasmic substances between animals immunized with 100 or 1,000 μg of cell

TABLE 1. *Volumes of 24-h skin test reactions in guinea pigs to selected test preparations after artificial immunization with 2,000, 1,000 or 100 μg of unextracted cell walls of H. capsulatum*

Immunizing dose (μg)	Volume of reaction in skin prepn: GP[a] mm³	GP[a] SE	HP[a] mm³	HP[a] SE	Cell sap[a] mm³	Cell sap[a] SE
2,000	11[b]	6	5	3	ND[c]	
1,000	58	5	0		36	31
100	83	35	5	5	128[d]	57

[a] GP, 50 μg per skin test dose; HP, 1:25; cell sap, 50 μg per skin test dose; PPD, 10 μg per skin test dose. SE, Standard error.

[b] Average based on six animals per group.

[c] ND, Not done.

[d] Skin-tested with SCS, 50 μg/ml, instead of cell sap.

walls is difficult, however, since the SCS used to skin-test those receiving 100 μg of cell walls was a more refined preparation than cell sap. None of the guinea pigs developed hypersensitivity detectable with HP regardless of the dose of cell walls used to immunize them.

Since 100 μg of cell walls appeared to be a suitable dose for immunization, five additional experiments were carried out in which the group size was seven, and the dose of cell walls used for immunization was 100 μg. In three of these experiments, the guinea pigs were skin-tested with GP and SCS either 1.5, 2.0, or 2.5 weeks after immunization, and in two of the experiments skin testing was done with RR as well. These data are collated in Fig. 2 and include 4-, 7-, 24-, and 48-h observations. Since each animal received two or three of the test preparations simultaneously, comparisons can be made of the relative activities of the three test antigens. SCS elicited the greatest reactions, GP elicited reactions less intense, and RR elicited the poorest reactions, barely within the weakly positive range. Hypersensitivity to GP was not demonstrable until 2.0 weeks after sensitization, whereas that to SCS was demonstrable by 1.5 weeks, albeit at a low level. RR was not tested at 1.5 weeks.

In a fourth experiment, guinea pigs were immunized with 100 μg of cell walls and weekly skin tests were administered for 4 weeks after immunization. Moreover, the reactions of such animals were compared with reactions in animals sensitized by infection (see below). In this experiment, group size was five guinea pigs. Therefore, 10 guinea pigs each were immunized with cell walls or infected with viable yeasts. One-half of each group was then skin-tested 1 and 3 weeks later, whereas the other half was skin-tested 2 and 4 weeks later. All animals were skin-tested with GP, SCS, and RR. The results of these skin tests are presented in Fig. 3. The maximum response to GP in animals sensitized with cell walls occurred 3 weeks after sensitization, whereas that to RR was consistently low but relatively constant from 2 to 4 weeks, and that to SCS seemed to increase steadily over the 4-week observation period. It is the data from the third week of this experiment that are presented in detail in Table 2.

MIF assays in artificially immunized guinea pigs. Since the direct method was employed, MIF assays were done only with artificially immunized animals; infected animals had been infected by the i.p. route and were considered unsuitable for the collection of peritoneal exudate cells.

Exudate cells from guinea pigs sensitized with 1,000 μg of cell walls were inhibited from migrating in the presence of GP and SCS, 70.5 and 78.4%, respectively. (The skin test data from these same animals are presented in Table 1.) Cells from guinea pigs sensitized with 100 μg of cell walls (Table 3) were inhibited also in the presence of GP and SCS. These data were derived from guinea pigs included in the experi-

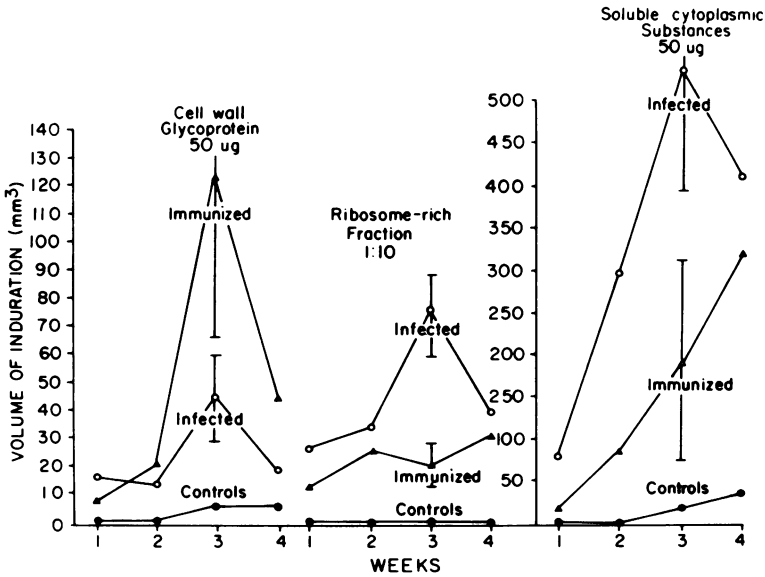

FIG. 3. *Twenty-four-hour skin test reactions in guinea pigs artificially immunized with 100 μg of cell walls of H. capsulatum or infected with 15 × 10⁷ yeasts when tested weekly for 4 weeks with three different components of H. capsulatum. I, Standard error of the mean.*

TABLE 2. *Diameter, thickness, and volume of induration of skin tests in guinea pigs 3 weeks after sensitization with 100 μg of H. capsulatum cell walls when skin-tested with selected preparations of H. capsulatum*

Skin test prepn	Skin test induration					
	Diameter[a] (mm)		Thickness[a] (mm)		Volume[a] (mm^3)	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
GP[b]						
Infected	10.6	0.9	0.33	0.07	44.9	15.4
Immunized	12.0	1.6	0.63	0.17	116.4	52.1
Control	4.0	1.3	0.14	0.05	4.0	1.3
SCS[b]						
Infected	20.4	1.5	1.11	0.15	479.2	127.2
Immunized	14.3	0.8	0.85	0.13	187.9	42.7
Control	3.5	2.1	0.14	0.08	7.3	5.6
RR[b]						
Infected	12.2	1.1	0.43	0.02	70.9	12.1
Immunized	8.5	0.6	0.28	0.06	22.8	8.4
Control	0		0		0	

[a] Diameter measured by palpation, thickness measured with dial calipers, and volume determined by the formula $V = 4/3\pi r^2 t$. SE, Standard error.

[b] GP, 50 μg per skin test dose; SCS, 50 μg per skin test dose; RR, 20 μg of protein per skin test dose.

TABLE 3. *Direct MIF assays using peritoneal exudate cells from guinea pigs 3 weeks after immunization with 100 μg of unextracted cell wall of H. capsulatum*

Antigen, concn (μg/ml)	Control[a]			Immunized		
	Area of migration		% Inhibition	Area of migration		% Inhibition
	mm^2	SE		mm^2	SE	
Experiment 1						
None	43.4	6.6		42.8	6.7	
GP, 10	44.1	4.8	0.0	32.6	1.3	33.9
SCS, 5	39.5	5.6	9.0	22.4	2.0	44.7
PPD, 5	40.7	4.4	6.3	25.9	1.5	39.5
Experiment 2						
None	67.6	5.3		48.6	7.3	
GP, 10	63.1	3.8	6.7	23.7	2.9	51.3
SCS, 10	65.3	5.3	3.5	13.9	1.7	71.4
PPD, 5	46.2	3.0	35.4	9.0	2.2	81.5

[a] Cells from animals to which nothing had been done before the injection of mineral oil. SE, Standard error.

ment in which weekly skin tests were performed (Fig. 3). However, the animals whose cells were used in the MIF assay had been separated at the beginning of the experiment and were never skin-tested. Since the guinea pigs were immunized with cell walls emulsified in FCA, purified protein derivative (PPD) was used as a positive control in the assay. Control cells should not have been inhibited in the presence of PPD because they were derived from animals to which nothing had been done before the injection of oil. Occasionally, however, control cells were inhibited, as can be seen in experiment 2. Such inhibition could have been due to a toxic effect of PPD at the concentration used or because, in fact, the animals were sensitive to PPD.

LT assays in artificially immunized guinea pigs. LT assays in which GP was used as an antigen were initially performed on pools of lymphocytes from animals immunized with cell walls. In those assays, lymphocytes from animals immunized with 1,000 μg of cell walls, as well as lymphocytes from those immunized with 100 μg, were stimulated in the presence of GP. The response, however, was greatest early and declined with time. For example, the E/C ratios at 2.5 and 3.5 weeks after sensitization with 1,000 μg of cell walls were 22.9 and 5.1, respectively, whereas those at 3.5, 4.5, and 5.5 weeks after sensitization with 100 μg of cell walls were 23.0, 7.0, and 4.5, respectively. E/C ratios derived from cultures stimulated with SCS were variable over the observation period, in that those lymphocytes from animals sensitized with 100 μg of cell walls, when tested 3.5, 4.5, and 5.5 weeks after immunization, had E/C ratios of 22.8, 5.2, and 12.2, respectively.

On the basis of the above data, it appeared that skin test reactivity to GP and SCS correlated with the level of reactivity to the same antigens in the LT assay. However, since the LT assays had been performed with pooled cells, skin test reactivity in individual animals could not be compared with LT data. There was always, therefore, the possibility that stimulation was occurring as the result of a mixed lymphocyte reaction, inasmuch as the guinea pigs were not syngenic. However, this possibility was considered remote since there was no evidence of stimulation in control cultures containing no antigen, or in any of the cultures containing lymphocytes from control animals. Nevertheless, to obtain more definitive data, four guinea pigs were immunized with 100 μg of cell walls, were tested in the LT assay individually on a weekly basis, and were skin-tested 3 weeks after sensitization. The results of this experiment (Table 4) showed there was, in fact, no correlation between the magnitude of the skin test response and the level of LT. Furthermore, only lymphocytes from two of the four animals transformed to a significant level with GP. Lymphocytes from three of the four animals transformed in the presence of SCS, but most of the responses did not occur until 5 weeks post-immunization.

Skin test reactions in infected guinea pigs. Two preliminary experiments were performed in which groups of six guinea pigs were infected with 10×10^7 and 6×10^5 yeasts, respectively, and then skin-tested 3 weeks later with GP and HP. Those infected with 6×10^5 were skin-tested also with crude cell sap. Those animals receiving 10×10^7 yeast cells were hypersensitive to GP and HP, and the reactions were similar in magnitude (Table 5). The most interesting reactions, however, occurred in those animals receiving 6×10^5 yeast cells. Hypersensitivity to HP was not demonstrable in them, but they did react strongly to crude cell sap. Their reactions to GP were in the weakly positive range.

In the experiment in which infected and artificially immunized animals were compared on a weekly basis for their cutaneous hypersensitivity to GP, RR, and the more refined SCS, the maximum response to all the preparations occurred 3 weeks after infection, and the responses to RR and SCS were better in infected than in artificially immunized animals (Fig. 3).

TABLE 4. *In vitro transformation of lymphocytes and skin test reactivity in individual guinea pigs at various times after artificial immunization with 100 μg of unextracted cell walls of H. capsulatum*

Test prepn	Animal no.	Transformation, E/C, at week:					Skin test (mm^3) week 3[b]
		1[a]	2	3	5	6	
GP	1	ND[c]	2.4	1.4	ND	ND	19
	2	0.7	2.0	2.7	2.0	ND	151
	3	ND	16.2	6.3	5.8	12.9	136
	4	3.6	2.5	1.5	5.4	4.9	28
SCS	1	ND	1.1	1.4	ND	ND	144
	2	1.4	1.0	1.4	5.8	ND	267
	3	ND	2.0	1.0	4.9	8.2	27
	4	4.0	2.2	2.0	3.9	7.8	76

[a] Weeks after immunization.
[b] Volume of 24-h reaction.
[c] ND, Not done.

TABLE 5. *Volumes of 24-h skin test reactions in guinea pigs to selected test preparations 3 weeks after infection with H. capsulatum yeasts*

Infecting dose	Volume of reaction in skin test prepn:					
	GP[a]		HP[a]		Cell sap[a]	
	mm^3	SE	mm^3	SE	mm^3	SE
10×10^7	42[b]	31	40	31	ND[c]	
6×10^5	19	7	4	3	83	37

[a] GP, 50 μg per skin test dose; HP, 1:25; cell sap, 50 μg per skin test dose. SE, Standard error.
[b] Averages based on six animals per group.
[c] ND, Not done.

LT assays with lymphocytes from infected animals. When lymphocytes were obtained from the guinea pigs described above, which had been infected with 6×10^5 yeasts, pooled, and tested with GP, they transformed well 2.5 and 3.5 weeks after infection; the E/C ratios were 44.3 and 12.4, respectively. There was a progressive decline from 2.5 to 4.5 weeks, however, so that by 4.5 weeks the level of transformation was minimal; i.e., the E/C ratio had dropped to 3.6. When lymphocytes from the guinea pigs infected with 15×10^7 yeasts were tested under similar conditions 3.5 weeks after infection, positive responses were observed in the presence of both GP and SCS, the E/C ratios were 5.5 and 7.4, respectively, but the responses were minimal or negative 2 weeks later.

As with artificially immunized guinea pigs, an attempt was made to determine if skin test reactivity could be correlated with levels of LT. Accordingly, eight guinea pigs were infected with 20×10^7 yeasts. They were all skin-tested 3 and 5 weeks later with GP, SCS, and HP, and 12 weeks later with only GP and SCS. Lymphocytes from four of the animals were tested in LT assays 1.5, 3.5, and 5.5 weeks postinfection, whereas those of the other four were tested at 2.5, 4.5, and 6.5 weeks, using GP and SCS as the test antigens. Data derived from both types of assays are presented in Table 6. The skin test data verified earlier observations with infected animals, in that hypersensitivity was observed when testing was done with both GP and SCS, but reactions to SCS were considerably greater. Moreover, maximum responses generally occurred 3 weeks after infection and were decreased by 5 weeks. The average response at 12 weeks was similar to that at 5 weeks, but there was a great deal of variation within the group. Again, HP elicited poor responses compared with SCS or GP, and, in fact, only three of the eight animals developed positive skin tests 5 weeks after infection, and those responses were minimal. Here, as with the artificially immunized animals, the level of lymphocyte transformation could not be correlated with the degree of hypersensitivity to the same test preparation. The greatest stimulation with both test preparations, however, occurred 3.5 and 4.5 weeks after infection and fell to insignificant levels by 5.5 weeks.

DISCUSSION

The evidence presented supports the theory that a cell-mediated immune response can be initiated by immunization with cell walls of *H. capsulatum*, inasmuch as a component of the

TABLE 6. *Comparison of 24-h skin test reaction with lymphocyte transformation in individual guinea pigs at various times after infection with 20 × 10⁷ yeasts of H. capsulatum*

Guinea pig no.	GP[a]				SCS[b]				HP skin test	
	Skin test		Transformation		Skin test		Transformation			
	Week[c]	mm³	Week	E/C	Week	mm³	Week	E/C	Week	mm³
1			1.5	1.5			1.5	4.5		
2				2.4				3.0		
3				5.7				8.0		
4				5.7				3.0		
5	3.0	47	2.5	7.5	3.0	236	2.5	2.8	3.0	3
6		75		1.3		268		1.0		51
7		63		1.3		228		1.3		2
8		63		4.0		397		6.7		21
1	3.0	63	3.5	3.8	3.0	144	3.5	10.7	3.0	19
2		34		3.6		174		3.0		13
3		47		15.3		150		8.9		6
4		236		4.2		378		2.7		26
5	5.0	8	4.5	7.6	5.0	133	4.5	5.8	5.0	0
6		ND[d]		ND		ND		ND		ND
7		0		5.3		95		8.5		14
8		58		8.6		221		9.5		10
1	5.0	30	5.5	1.9	5.0	185	5.5	2.1	5.0	1
2		13		2.8		133		2.2		7
3		52		0.9		136		1.0		3
4		51		0.8		221		1.0		37
5			6.5	0.5			6.5	0.6		
6				ND				ND		
7				1.1				2.3		
8				1.2				1.3		
1	12.0	24			12.0	212				
2		0				62				
3		1				57				
4		106				348				
5	12.0	2			12.0	8				
6		ND				ND				
7		37				114				
8		8				114				

[a] In transformation assays, 50 μg/ml was used; 50 μg/dose was used in skin testing.
[b] In the transformation assays, 10 μg/ml was used; 50 μg/dose was used in skin testing.
[c] Weeks postinfection.
[d] ND, Not done.

cell wall, GP, elicited cutaneous reactions in vivo by skin testing, and cells isolated from such animals reacted with the appropriate responses in vitro in the MIF and LT assays. Animals infected with viable yeasts were primed to react to GP as well, so that when skin-tested with it they developed positive reactions and their lymphocytes transformed in its presence. Artificially immunized animals developed greater levels of cutaneous hypersensitivity to the GP than infected animals, however. It is possible (and probable) that the skin test response to GP was a mixture of immediate and delayed hypersensitivity, and that the artificially immunized animals, at the time the skin test was performed, had a component of immediate hypersensitivity overlaying that of the delayed, making the overall reaction larger.

Of interest, also, was the fact that guinea pigs immunized with cell walls, even those immunized with as little as 100 μg, responded well to skin testing with SCS, although not as well as infected animals. There are two possible

explanations for such a response in the artificially immunized animals. (i) Segments of the wall large enough to be immunologically active may be manufactured in the cytoplasm and present in the SCS in sufficient quantity to detect the immune response to them. (ii) On the other hand, the cell walls used for immunization may have been contaminated with proteins from the cytoplasm, and skin-testing with SCS then detected an immune response to the contaminants. This possibility is presently under investigation, but even if the stimulation was a result of contamination, the data become more intriguing because this would mean that the immunological response would have been directed to only a small amount of material, probably less than 5 to 10 μg. Such an immunogen would perforce have been potent in order to stimulate the response to the level observed. SCS was, in general, a highly reactive antigen, and, on the basis of protein concentration, was more reactive than HP when the two were compared as skin-testing antigens. This was illustrated in the case of animals infected with only 6×10^5 yeasts, where that dose was insufficient for estabishing sensitivity demonstrable by skin testing with HP, but was adequate for inducing sensitivity demonstrable with SCS. Since there was no attempt made to recover viable *H. capsulatum* from the guinea pigs after infection, it could be argued that 6×10^5 yeasts were simply not sufficient for establishing an infection and were killed virtually immediately by macrophages attracted to the perimediately by macrophages attracted to the peritoneal cavity, and that the net result was immunization with dead cells rather than infection with viable cells, the number of dead cells being sufficient for inducing delayed hypersensitivity to SCS but not capable of inducing delayed hypersensitivity to HP. Furthermore, it may be that two different components or products of *H. capsulatum* were responsible for the cutaneous reactions observed to SCS and HP. SCS elicited positive cutaneous reactions in both infected and artificially immunized guinea pigs, whereas HP detected sensitivity in only infected guinea pigs. The fact that a positive cutaneous reaction was obtained with SCS corroborates the findings of Salvin and Ribi (18) who reported that a soluble cytoplasmic fraction elicited a response in infected guinea pigs, which, on the basis of dilution experiments, was somewhat better than the response elicited with cell walls. In contrast to that, however, Dyson and Evans (7) were not successful in using somatic antigens for skin testing.

Overall, GP appeared to be a less effective antigen than SCS, but two considerations may alter the assessment of its activity. First, nothing is known at this time of the specificity of each of the antigenic preparations, and GP may have, in fact, a higher degree of specificity than SCS. Second, both are crude preparations, and when the active ingredient(s) is separated from the inactive portions, GP may prove to be a more effective antigen. An additional piece of evidence that tends to discredit GP as a suitable skin-testing antigen, however, was the fact that only three of seven animals skin-tested 3 months after infection displayed hypersensitivity to that antigen, whereas reactions in the same animals to SCS were large, with only one exception.

Other investigators have used antigens of *H. capsulatum* in vitro for analyses of cell-mediated immune responses (e.g., 15, 22, 1, 16), but the antigen was either HP or some other antigen of mycelial origin. Although HP has been a useful epidemiological and clinical tool, it is not without its difficulties, viz., cross-reacting responses may confuse the interpretation of results, and reproduction of a standard HP with known potency is a problem (10, 13), so that a more specific and easily reproducible antigenic preparation would be desirable.

The experiments described here emphasize the need for careful design in investigations where the effect of a specific immunological state is being assayed, because both the dose of immunizing antigen and the time at which the assays are performed determine, in part, the observed effect. For example, 2 mg of cell walls, even when given in adjuvant, seemed to inhibit delayed hypersensitivity, whereas 100 μg of the same was excellent for establishing delayed hypersensitivity, but the response peaked at 3 weeks and then declined. Feingold et al. (8), working with protein antigens, obtained results that indicated that the type of skin reactivity induced in guinea pigs was dependent on the sensitizing doses of the antigens. They found, as we did here, that high doses of antigen suppressed delayed hypersensitivity and enhanced immediate-type hypersensitivity. Additionally, although it was not the intent of this investigation to do a careful study of the effect of different doses of viable yeasts on cell-mediated immune responses, it appears from the limited observations made that the number of yeasts employed for infection determines, at least in part, subsequent results. For example, 6×10^5 was not sufficient for stimulating a response demonstrable by skin testing with HP, whereas 20×10^7 did stimulate a response to HP, but it was maximum at 3 weeks and reduced by 5 weeks.

Finally, in the experiments in which both infected and artificially immunized animals were tested individually in the LT assay, there was no correlation between the level of stimulation and the degree of hypersensitivity. Bice et al. (2), working with humans and *Candida* antigens, did find such a correlation.

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