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Allergenic and Blastogenic Reactivity of Three Antigens from *Mycobacterium tuberculosis* in Sensitized Guinea Pigs

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Three antigens from a culture filtrate of *Mycobacterium tuberculosis* H37Rv were purified by affinity chromatography, using monoclonal antibodies. The molecular weights of the purified antigens are 17,000 to 19,000, 32,000 to 33,000, and 39,000, respectively, and by their migration patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions, they all appeared to be single-chain polypeptides. Western blot and enzyme-linked immunosorbent assay analyses indicated that the antigens are non-cross-reactive. All antigens generated an intermediate to strong skin reaction when tested in guinea pigs previously immunized with a live *M. bovis* BCG vaccine or with an oil emulsion preparation of phenol- or heat-killed *M. tuberculosis*. Lymphocytes isolated from peripheral blood or lymph nodes of similarly immunized guinea pigs could be stimulated by purified protein derivative and the purified antigens. Qualitative differences in stimulatory capacity between the preparations were demonstrated. The antigens may prove useful in further studies of the immunology and pathogenesis of tuberculosis.

Many attempts have been made to purify antigenic components of *Mycobacterium tuberculosis* by conventional biochemical techniques (2). Such methods are often complicated and tedious, eventually resulting in only low yields, and usually it is not possible to reach a sufficiently high degree of purification. By application of the hybridoma technique to monoclonal antibody (MAb) production (4), it has become possible to purify antigens in single-step procedures, using MAb in immunosorbent techniques.

By using three MAb prepared in our laboratory (7), corresponding antigens from a culture filtrate of *M. tuberculosis* H37Rv have been purified by affinity chromatography. The three antigens have been characterized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their reactivity has been studied further by immunoblotting and in an enzyme-linked immunosorbent assay (ELISA). Finally, the delayed-type hypersensitivity skin test activity of the purified antigens in sensitized guinea pigs was assessed, and the antigens were used to stimulate lymphocytes isolated from such guinea pigs.

MATERIALS AND METHODS

Preparation of CF. Culture filtrate (CF) of *M. tuberculosis* H37Rv was prepared according to standard procedures. In brief, the bacteria were grown on Sauton medium and were separated from the CF by filtration. The filtrate was precipitated twice with ammonium sulfate. Following centrifugation, the supernatant was dialyzed against phosphate-buffered saline, pH 7.4, and passed through a 0.22-μm sterile filter. The protein concentration of the CF was adjusted to 5 mg/ml as measured spectrophotometrically by A_{280}. The antigen preparations were stored at −20°C.

Production of MAb. MAb HYT 6, HYT 27, and HYT 28 were produced as previously described (7). All antibodies belonged to the immunoglobulin G1 subclass and κ subtype.

Purification of MAb. Ascites fluids from all three MAb were purified on a protein A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). For HYT 6 MAb, commercial binding and elution buffers (Bio-Rad Laboratories, Richmond, Calif.) were used, and for HYT 27 and HYT 28 MAb the ascites fluids were diluted in phosphate buffer (0.1 M; pH 8) and eluted with 0.1 M glycine hydrochloride buffer, pH 2.8. HYT 27 MAb were further purified by anion-exchange chromatography (mono-Q, fast protein liquid chromatography equipment; Pharmacia).

Preparation of affinity columns. HYT 6 MAb was coupled to Mini-Leak vinylsulfone agarose (Kem-en-tec, Hellerup, Denmark), and HYT 27 and HYT 28 MAb were coupled to CNBr-activated Sepharose 4B (Pharmacia). All MAb were coupled to the matrices in a concentration of 2 mg/ml following the procedures suggested by the manufacturers, and 90 to 100% of the MAb were coupled for all columns prepared.

Affinity purification of antigen. The mycobacterial CF was diluted to 1 to 5 mg/ml in a washing buffer (phosphate-buffered saline, pH 7.4, with 0.5 M sodium chloride and 0.05% Tween 20) and passed through the affinity columns at a flow rate of approximately 25 ml/h. The amount of filtrate passed was 20 to 100 mg. The chromatography was run at room temperature (HYT 27 and HYT 28) or 4°C (HYT 6) with no incubation. After being washed with at least 10 times the bed volume, the bound antigens were eluted with 0.1 M glycine hydrochloride in washing buffer, pH 2.8. With an ELISA system it was found that 0.1 M glycine hydrochloride, pH 2.8, was an excellent eluting buffer for the three HYT antigens in question (B. Sørensen, personal communication). Positive fractions as assessed by ELISA were pooled and dialyzed against phosphate-buffered saline, pH 7.4. The protein contents were measured in a spectrophotometer by A_{280}. The eluates were stored at −20°C.

SDS-PAGE and immunoblotting. SDS-PAGE was carried out in a discontinuous system (6), using a 12.5% (wt/vol) acrylamide gel or a gradient of 10 to 20% (wt/vol) acrylamide for the separation. The antigens were transferred onto nitrocellulose paper by either the conventional Western blotting (8) or the semidry blotting (semidry electroblotter A; Ancos, Ølstykke, Denmark) technique (5).

Incubation of the nitrocellulose paper with antibodies was performed as described elsewhere (1). When mouse MAb

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were used for the primary incubation, the secondary antibody was a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (P 260; DAKOPATTS, Glostrup, Denmark). When the polyclonal antibodies were used for the incubation, an HRP-conjugated swine anti-rabbit immunoglobulin was used as secondary antibody (DAKOPATTS P 217).

Antigen recovery after purification by affinity chromatography. Microtiter immunoplates, 96 wells (Nunc, Roskilde, Denmark), were coated with the protein A-purified MAb, incubated for 2 h at room temperature, and subsequently blocked by incubation for 1 h with 1% (wt/vol) bovine serum albumin. Threefold serial dilutions of CF, effluent, or eluate were incubated overnight at 4°C. A polyclonal rabbit anti-*M. tuberculosis* immunoglobulin was used as detecting antibody. Following 2 h of incubation at room temperature, HRP-labeled swine anti-rabbit immunoglobulin (DAKOPATTS P 217) was added, and the plates were incubated for 1 h at room temperature. Orthophenylenediamine (Kem-entec) was added to the wells, and the color reaction was read at A_{490}.

Cross-reactivity among HYT antigens. A possible cross-reactivity among the HYT antigens was tested by immunoblotting and ELISA. The immunoblotting was performed as described above. In the ELISA, a polyclonal rabbit anti-*M. tuberculosis* immunoglobulin was used as the "capture" antibody. The second layer was HYT 6, HYT 27, or HYT 28 reactive eluates added in serial dilutions (twofold). The reactivity of the bound HYT antigens towards the HYT antibodies was tested in all antigen/antibody combinations. The color reaction was developed as described above. The ELISA was done in a reverse fashion, too, by coating the immunoplates with the MAb.

Biological activity of HYT antigens. Skin test and lymphocyte stimulation (LS) test methods have been given previously (3). These are briefly reported here.

(i) Guinea pigs and immunization. Guinea pigs, strain Ssc:A1, weighing 500 to 600 g were used. The guinea pigs were immunized by four intradermal injections in the abdomen with 0.1 ml of the chosen immunogen. The immunization time was 4 weeks.

(ii) Immunogens. The following immunogens were used: standard living *M. bovis* BCG vaccine (Statens Seruminstitut, Copenhagen, Denmark); phenol-killed *M. tuberculosis* H37Rv; working cultures (WC) of *M. intracellulare* (WC2), *M. kansasii* (WC3), or *M. scrofulaceum* (WC4), suspended in paraffin oil (semidry weight, 0.4 mg/ml); heat-killed *M. tuberculosis* in paraffin oil (TB) (dry weight, 0.4 mg/ml); paraffin oil alone (M52).

(iii) Antigens. Tuberculin purified protein derivative (PPD) RT23 and sensitins RS23 (*M. intracellulare*), RS30 (*M. kansasii*), and RS95 (*M. scrofulaceum*) were used for skin tests, and tuberculin PPD RT40 was used for LS tests (Tuberculin Department, Statens Seruminstitut). Affinity-purified preparations of HYT antigens for skin tests were HYT 28 (195 μg/ml), HYT 27 (75 μg/ml), and HYT 6 (83 μg/ml); for LS tests, these were HYT 28 (172 μg/ml), HYT 27 (75 μg/ml), and HYT 6 (83 μg/ml). Dilutions indicated in Results were prepared from these basic materials. The indicated negative control was an eluate from an affinity column without application of antigen.

(iv) Skin test. Guinea pigs were given intradermal injections of 0.1 ml of the preparations. Reactions were read after 24 h by two independent readers, each measuring two transverse diameters of the erythema. Reaction diameters are given as means corresponding to single diameters.

Guinea pigs immunized with BCG, TB, or H37Rv were skin tested with undiluted, 1:2- and 1:4-diluted HYT antigen and with RT23 at 0.3, 0.6, and 1.2 μg. Guinea pigs immunized with WC2, WC3, or WC4 were skin tested with undiluted and 1:2-diluted HYT antigen, with RT23 at 0.6 and 1.2 μg, and with 1 or 0.5 μg of the RS antigens pertaining to the heterologous or homologous species, respectively.

(v) LS test. Peripheral blood lymphocytes (PBL) were isolated in a density gradient and washed twice. The cell concentration was adjusted to 2×10^6 viable cells per ml. Lymph node lymphocytes (LNL) were isolated from the inguinal and axillary lymph nodes and washed twice, and the cell concentration was adjusted to 4×10^6 viable cells per ml. A 0.1-ml portion of the cells was cultured with 0.1 ml of antigen or mitogen for 5 days; 1 μCi of [^{3}H]thymidine was added during the last 22 h. All tests were carried out in triplicate, and the cells were either left unstimulated or stimulated with tuberculin PPD (final concentrations, 64, 16, 4, and 1 μg/ml) or HYT antigens in final dilutions of 1/8, 1/32, 1/128, and 1/512. Results are expressed in counts per minute as the geometric mean of triplicate cultures. The calculations were performed by using the logarithm of the geometric means.

Statistical methods. (i) Skin test results. Standard deviations corresponding to variations between animals have been calculated for each antigen-immunogen combination. It was found acceptable to use common standard deviations for groups of immunogens (given in Table 1). Standard errors for a difference [SE(d)] between mean reactions to an antigen for two immunogens can be calculated as follows:

$$SE(d) = \sqrt{(SD_1^2/n_1) + (SD_2^2/n_2)}$$

where n_1 and n_2 are the numbers of guinea pigs. For comparisons between antigen reactions for a particular immunogen, the interaction between animals and antigen reactions was taken into account by the use of a two-way analysis of variance.

(ii) LS results. For each immunogen and each cell type, a two-way analysis of variance was carried out by using a missing-plot technique. In all cases, clearly significant variations between animals were found. The analysis of variance performed on the results for the chosen dose of the five antigens showed a significant interaction between antigens and animals, with variances showing some variations with the immunogen and a tendency towards higher variances for PBL than for LNL. The highest variance estimate found for each of these two types of cells was used for calculation of the standard errors corresponding to the difference between two mean log counts per minute.

RESULTS

Isolation and characterization of purified proteins. Crude CF and eluates from the HYT affinity columns were run on SDS-PAGE under reducing conditions and tested in immunoblotting. Nitrocellulose sheets with crude CF and eluates were incubated with different antibodies as illustrated in Fig. 1. Lanes 1, 2, and 3 show eluates HYT 6, HYT 27, and HYT 28 incubated with polyclonal rabbit anti-*M. tuberculosis* antibody. For these eluates, single bands are seen at 17 to 19, 32 to 33, and 39 kilodaltons, respectively. In some experiments, a double band at 32 to 33 kilodaltons is seen in the HYT 27 eluate. When incubated with the corresponding MAb, a similar picture is seen (lanes 4 to 6).

A chromatographed crude CF from *M. tuberculosis* H37Rv is shown in lanes 7 to 10: incubation was with MAb

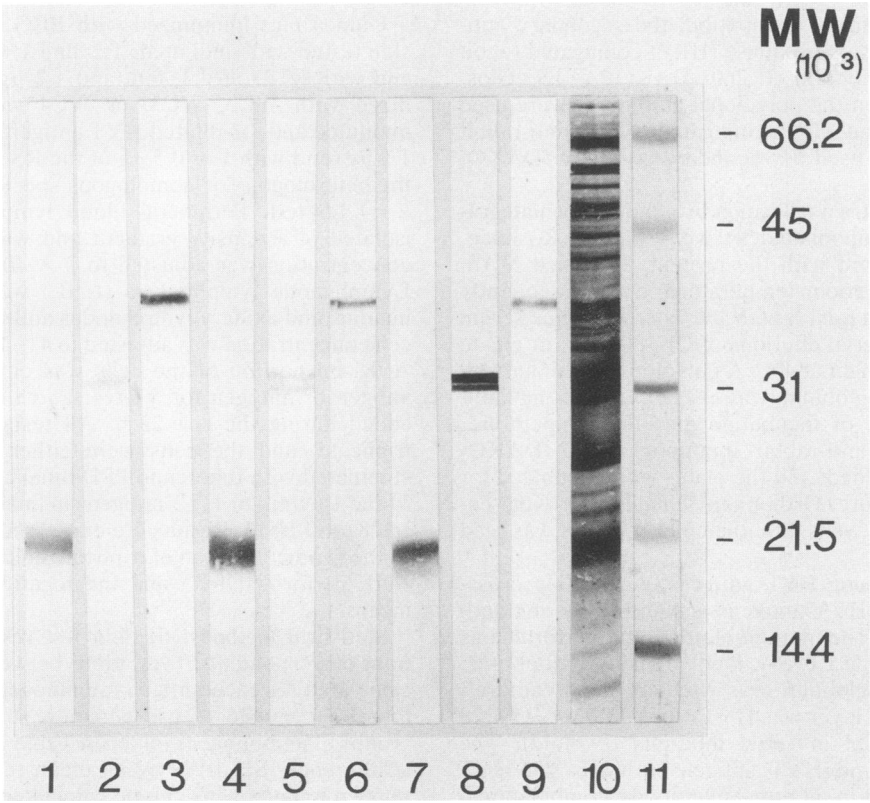

FIG. 1. Characterization of eluates from HYT 6, HYT 27, and HYT 28 affinity columns. Following transfer of the protein onto nitrocellulose paper, the lanes were incubated with polyclonal rabbit anti-*M. tuberculosis* antibody (lanes 1 to 3 and 10), HYT 6 MAb (lanes 4 and 7), HYT 27 MAb (lanes 5 and 8), or HYT 28 MAb (lanes 6 and 9). The bands were subsequently stained with HRP-labeled secondary antibody. Chromatographed antigens were HYT 6 eluate (lanes 1 and 4), HYT 27 eluate (lanes 2 and 5), HYT 28 eluate (lanes 3 and 6), and H37Rv CF (lanes 7 to 10). Molecular weight (MW) markers were visualized in amido black (lane 11) and are, top to bottom, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. For experimental details, see Materials and Methods.

HYT 6, HYT 27, and HYT 28 and a polyclonal rabbit anti-*M. tuberculosis* antibody. The last shows the high degree of heterogeneity of the crude filtrate before affinity purification and illustrates the very high activity of the polyvalent rabbit antiserum against mycobacterial antigens.

Recovery of antigen from affinity chromatography purification. Serial dilutions of the eluates and effluents were compared with dilutions of the crude CF in a "capture ELISA." The percent recovery of specific antigen in the eluates was estimated from 50% of maximum optical density. An example of a recovery estimate for the HYT 6 antigen is given in Fig. 2. The recovery can be calculated to 85%, and when corrected for volume differences, the yield was estimated to be around 40%.

From a series of 15 experiments, the recovery varied between 10 and 60% for antigen HYT 6 purification.

Estimation of recovery for HYT 27 and HYT 28 antigen purification gave varying results. Capture ELISAs were tried with polyclonal antibody- or MAb-coated microtiter plates. In most experiments the slopes were found to be so different from each other that calculation of relative amounts by using a parallel line assay was unjustified. Thus, it was not possible to quantify the recovery by this method.

For HYT 6 antigen, the total amount of protein in the eluate, relative to the total amount in the crude CF, varied from 0.3 to 2% when estimated from absorption spectrophotometry at A_{280}. The recoveries estimated by A_{280} absorption relative to the total amount of protein in HYT 27 and HYT 28 purifications were 5 to 14% and 1 to 3%, respectively.

Cross-reactivity. To analyze possible cross-reactivities among the purified antigen components from the H37Rv CF, a capture ELISA was performed. No cross-reactivity was found between the eluates when polyclonal anti-*M. tuberculosis* immunoglobulin was used for coating the ELISA plates (Fig. 3). The low homologous binding seen for the HYT 6 antigens is discussed below.

These results correlate well with the results found for eluates tested in immunoblotting and incubated with the various MAb. No bands were seen stained unless incubation was with the homologous MAb (data not shown).

Immunoblots of the eluates were incubated with polyclonal rabbit antisera directed against different mycobacterial species. All HYT antigens reacted with *M. tuberculosis* and BCG antisera, and none of the antigens reacted with *M. marinum* or *M. scrofulaceum* antisera. HYT 6 antigen reacted with *M. avium*, *M. intracellulare*, and *M. kansasii*, while HYT 27 antigen showed reactivity towards *M. avium*, *M. intracellulare*, and *M. fortuitum* (results not shown).

Biological activity of the antigens in guinea pigs. Skin reactions to selected doses of HYT antigens, of tuberculin PPD RT23, and of the mycobacterial sensitins are shown in Table 1. It appears that all HYT antigens had a strong skin test activity in guinea pigs sensitized with BCG or with the *M. tuberculosis* preparations TB and H37Rv. HYT 6 antigen gave stronger reactions in the BCG group than in the TB and H37Rv groups. For the HYT 27 and HYT 28 antigens, the situation was reversed, with strong reactions in the two latter groups. Compared with the HYT 27 and HYT 28

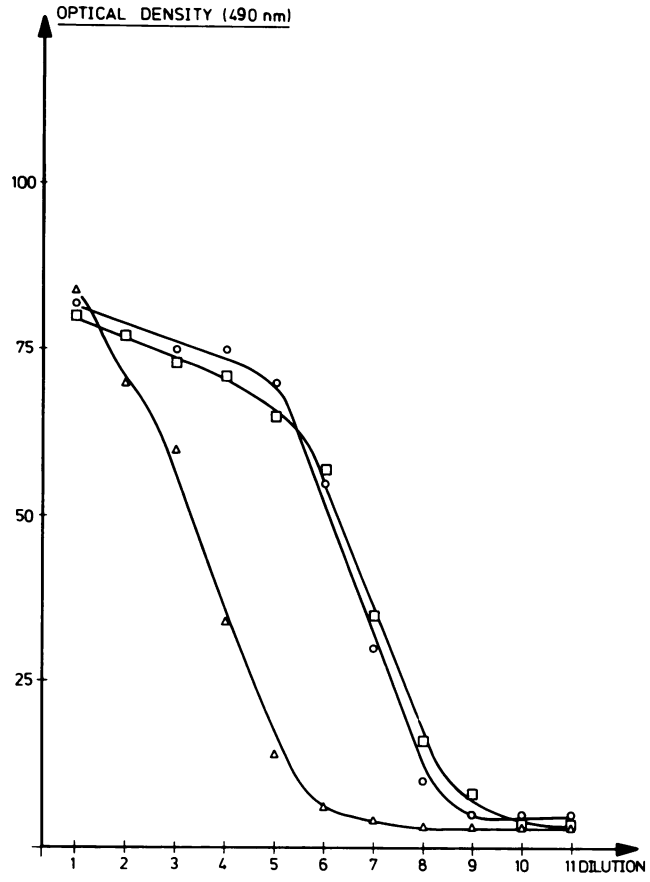

FIG. 2. Estimation of reactive protein towards HYT 6 MAb in filtrate H37Rv CF, effluent, and eluate fractions after affinity chromatography on HYT 6 MAb Mini-Leak vinylsulfone agarose column. Serial dilutions (threefold) of crude CF (32 ml; □), the total effluent fraction (57 ml; △), and the eluted fraction (40 ml; ○) were titrated in the antigen-capture ELISA. Estimated recovery of HYT 6 MAb-reactive H37Rv protein was 40% in the eluate.

antigens, the reactions of RT23 were relatively stronger in the BCG and TB groups than in the H37Rv group.

In the guinea pigs immunized with "atypical" mycobacteria, HYT 6 antigen and RT23 gave negative skin reactions, whereas HYT 27 and HYT 28 antigens elicited a weak to intermediate skin reaction in these three groups of guinea pigs. The reactions to the homologous (with respect to the *Mycobacterium* species) sensitins were always stronger than the reactions to the heterologous sensitins.

LS activity of HYT antigens. PBL and LNL isolated from immunized guinea pigs were stimulated with a control buffer, the HYT antigens, or PPD. LS results for the antigen concentration, which was optimally stimulating in most immunogen-cell type combinations, are shown in Table 2. None of the antigens stimulated cells from the guinea pigs given M52. Tuberculin PPD RT40 strongly stimulated lymphocytes from all immunized guinea pigs. HYT 6 antigen had, with the exception of the TB/PBL combination, a lower but otherwise similar stimulatory capacity. HYT 28 antigen stimulated cells from the TB and H37Rv groups, and HYT 27 antigen stimulated cells from the H37Rv group only.

DISCUSSION

It has been possible to purify three antigens from mycobacterial CF by a simple one-step procedure, using MAb as immunosorbents in affinity chromatography. The three antigens have molecular weights of 17,000 to 19,000 (HYT 6), 32,000 to 33,000 (HYT 27), and 38,000 (HYT 28). The proteins appear to be single-chain polypeptides since they migrated to the same position in the gels under reducing and nonreducing conditions (data not shown).

By Western blotting, it was demonstrated that a high degree of purification was obtained by affinity chromatography. The three fractions, the crude CF, the effluent, and the eluate, were tested by incubation with both MAb and polyclonal antibodies towards *M. tuberculosis*. No irrelevant bands were detected even when the highly active polyvalent antiserum was used.

As mentioned in Results, the recovery of antigen was found to vary from one experiment to another, despite standardized purification procedures. We cannot at present explain these divergences.

Young et al. (9) find a recovery of 0.7% of total protein in eluate TB71 (a 38-kilodalton protein from *M. tuberculosis* possibly similar to our HYT 28 antigen). This result correlated well with our own findings (3%) for this molecule.

Recovery as measured by titration of antigen in a trapping ELISA showed that it was possible to recover 10 to 60% of the HYT 6-defined reactivity originally found in the filtrate. For purification of HYT 27 and HYT 28 antigens, it was not possible to obtain reliable recovery estimates by using the capture ELISA system. This was because the titration

TABLE 1. Skin test activity of HYT antigens in guinea pigs immunized with mycobacterial immunogens

Skin test antigen[a]	Mean reaction diam (mm)[b] with given immunogen						
	BCG	TB	H37Rv	*M. intracellulare* WC2	*M. kansasii* WC3	*M. scrofulaceum* WC4	None
HYT 6	14.7	11.1	9.8 (1.33)[c]	3.3	2.8	3.1 (0.56)	4.6 (0.56)
HYT 27	12.7	15.4	14.7 (1.33)	7.6	7.5	6.3 (3.50)	4.1 (1.23)
HYT 28	12.0	14.8	15.0 (1.34)	9.0	6.8	5.8 (2.85)	3.4 (1.20)
RT23	13.0	12.7	10.8 (0.85)	3.1	3.2	3.3 (1.03)	2.9 (1.03)
RS23 (*M. intracellulare*)	ND[d]	ND	ND	**6.7**[e]	4.5	5.4 (2.93)	ND
RS30 (*M. kansasii*)	ND	ND	ND	3.9	**6.8**	4.4 (2.06)	ND
RS95 (*M. scrofulaceum*)	ND	ND	ND	5.2	4.2	**8.5** (2.96)	ND

[a] Skin test doses in 0.1 ml: HYT antigens, 1:1 dilution; RT23, 0.6 μg; RS preparations, 1 μg.
[b] Means of five (BCG, TB, H37Rv, WC3, and "None") or four (WC2 and WC4) immunized guinea pigs.
[c] Numbers in parentheses are standard deviations common for the preceding immunogen(s); see Materials and Methods.
[d] ND, Not done.
[e] Boldface values indicate skin reactions to homologous sensitins. These reactions were significantly different ($P < 0.05$) from reactions to the heterologous sensitins.

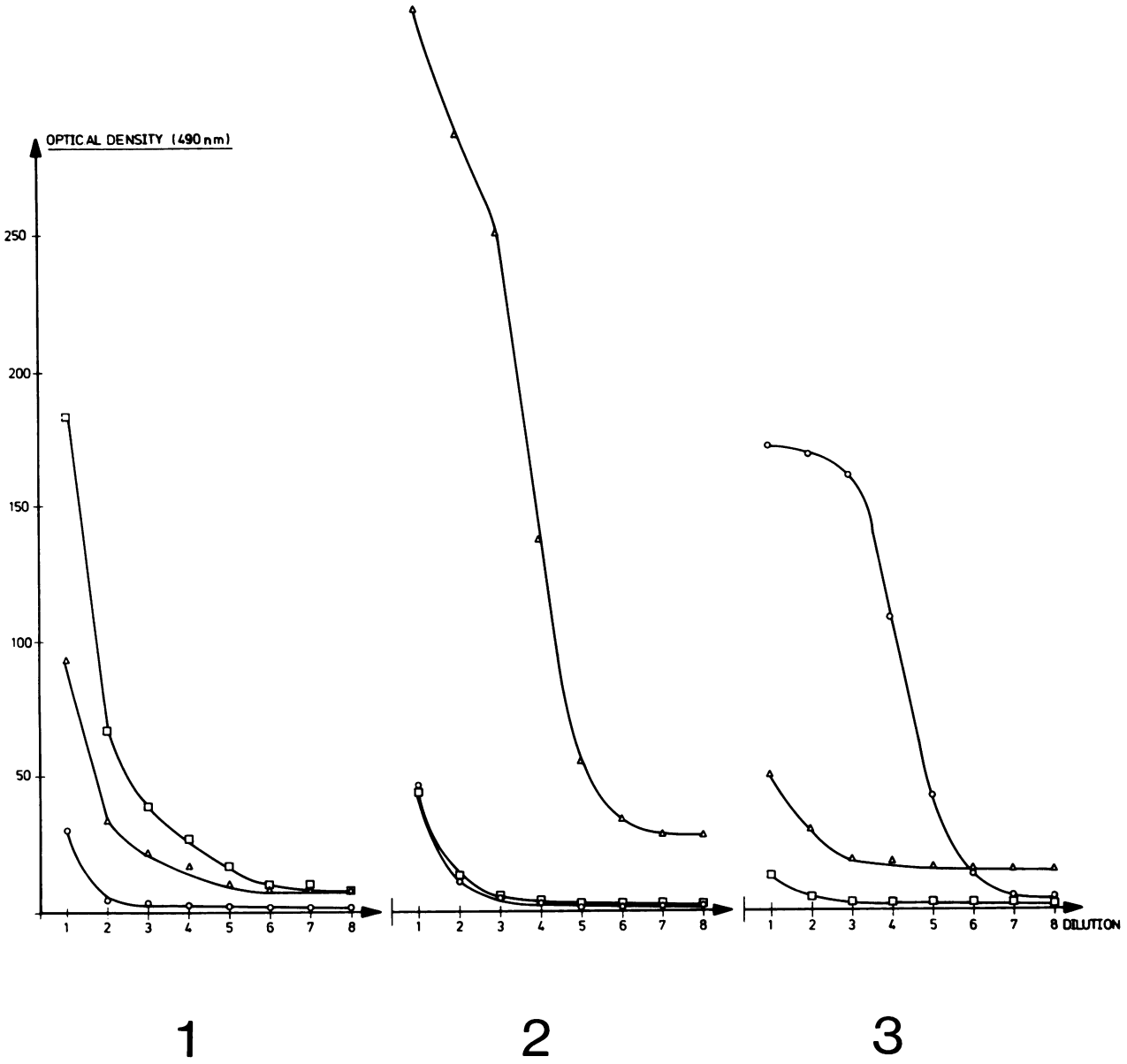

FIG. 3. Assay of cross-reactivity between affinity chromatography-purified (1) HYT 6-, (2) HYT 27-, and (3) HYT 28-reactive H37Rv proteins. Serial dilutions (twofold) of the antigens were added to immunoplates coated with rabbit anti-*M. tuberculosis* antibody. Caught H37Rv protein was identified by MAb HYT 6 (□), HYT 27 (△), and HYT 28 (○). Bound mouse antibody was detected by HRP-labeled rabbit anti-mouse immunoglobulin antibody.

curves for the starting materials and the purified antigens were not parallel. The reason for this remains obscure, but possible minor structural changes of the antigens induced during the purification procedures might be the cause.

To demonstrate that the three MAb react with three non-cross-reactive epitopes on the molecules in question, also under nondenaturing conditions, a capture ELISA was carried out. Bound HYT antigen was identified by means of the homologous HYT MAb as detecting antibody. When irrelevant MAb were used to detect antigen, only minor reactivities were seen, which could be due to unspecific binding. This hypothesis is also supported by the finding that such reactivities to the heterologous MAb could not be detected in immunoblotting. It was impossible to obtain a high antigen binding for HYT 6 antigen (Fig. 3), despite considerable efforts to optimize the ELISA system. Several reasons for this can be suggested: low quantities of the relevant antibody in the polyvalent serum, low affinity of the anti-HYT 6 antibody for the plastic surface, or conformational changes of the antibody due to binding to the plastic surface.

The biological relevance of the purified HYT antigens has been demonstrated by positive skin and LS tests in guinea pigs sensitized with mycobacterial antigens. Comparisons of skin and LS test results revealed some dissociations between the methods in the estimation of biological activity of the HYT antigens. All HYT antigens gave positive skin reactions in groups of guinea pigs immunized with BCG or the *M. tuberculosis* preparations. Conversely, HYT 27 antigen did not stimulate cells isolated from BCG- or TB-immunized

TABLE 2. Blastogenic activity of HYT antigens in cultures of PBL and LNL from guinea pigs immunized with mycobacterial immunogens

Stimulant[a]	Activity (cpm)[b] with given immunogen							
	BCG		TB		H37Rv		M52	
	PBL	LNL	PBL	LNL	PBL	LNL	PBL	LNL
Medium control	150	3,600	130	4,600	160	3,800	180	5,900
Control buffer	400	5,000	300	5,900	700	7,100	130	5,800
HYT 6	1,000[c]	13,000[c]	300	19,000[c]	10,000[c]	42,000[c]	160	6,800
HYT 27	600	500	300	8,000	2,000[c]	14,000[c]	250	6,200
HYT 28	500	5,000	700[c]	12,000[c]	7,900[c]	35,000[c]	170	5,000
PPD	32,000[c]	60,000[c]	41,000[c]	98,000[c]	42,000[c]	79,000[c]	260	6,200

[a] HYT antigen concentrations were 1:32 dilutions of the values given in Materials and Methods. The PPD concentration was 4 μg/ml.
[b] Means of five (BCG, TB, and H37Rv) or four (M52) guinea pigs.
[c] Significantly different from the value of the control buffer, $P < 0.05$. The standard errors ($n = 5$) corresponding to differences between two mean log counts per minute were 0.180 and 0.145 for PBL and LNL, respectively.

guinea pigs and HYT 28 antigen did not stimulate cells from the BCG group. Because of the consistently high reactivity in all groups to tuberculin PPD and because all HYT antigens stimulated cells from at least one group, these differences did not reflect a lack of sensitivity of the LS system, but rather differences in the biological role and significance of the HYT antigens. That HYT 6 antigen stimulated LNL but not PBL from the TB group may have a similar explanation.

The biological activity of a 38-kilodalton antigen, possibly similar to ours, has previously been demonstrated by skin tests in guinea pigs sensitized with BCG or *M. tuberculosis* and by LS tests with PBL from TB patients or BCG-vaccinated healthy individuals (9).

The antigen doses used in the LS system were of similar magnitude (2 to 5 μg/ml) for all antigens, including PPD. Despite this, very large differences in stimulating activity and in the shapes of the dose-response curves were demonstrated both within and between the immunization groups. In the skin test system, the tuberculin RT23 dose was 0.6 μg and the HYT antigen doses were 4 to 10 μg. Skin reactions of comparable magnitude were obtained with RT23 and the HYT antigens despite the factor 10 difference in order of magnitude. Thus, the superiority of tuberculin PPD over the HYT antigens in stimulating capacity was seen in both experimental systems. The explanation is possibly the multiplicity of antigens in tuberculin PPD which stimulate a wide range of T cells with different specificities and biological effects.

HYT 6 antigen did not induce skin reactions in guinea pigs immunized with atypical mycobacteria. A previous report from our laboratory demonstrates a reactivity of HYT 6 MAb to *M. intracellulare* and *M. kansasii* by immunoblotting (1). A converse picture was seen for HYT 28 antigen. This antigen did elicit skin reactions in guinea pigs immunized with atypical mycobacteria despite the fact that the antigen was not demonstrated in these mycobacterial species by MAb HYT 28 or different mycobacterial antisera in immunoblotting. Consequently, with respect to mycobacterial species, the patterns of specificity for the T- and B-cell epitopes seem to differ for these antigens.

By affinity chromatography, three antigens from *M. tuberculosis* CF have become available in a highly purified form. The results obtained in the present study are a step towards a better identification and characterization of the antigens, which are constituents of the tubercle bacillus and are possibly relevant for the understanding of the pathogenesis of tuberculosis. These purified antigens are being used in studies on distribution, specificity, and cross-reactivity of mycobacterial T- and B-cell epitopes on single mycobacterial antigens.

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