□ 원 저 □

A Study of Cell Surface Antigenic Relationships Among Mycobacteria by Enzyme-Linked Immunosorbent Assay

Korean Institute of Tuberculosis, Korean National Tuberculosis Association

S.J. Kim, G.H. Bai, S.H. Lee and Y.P. Hong

*Central Research Laboratory, Seoul National University Hospital, Seoul, Korea

H.H. Kwon

=국문초록=

酵素結合免疫分析法(ELISA)을 이용한 抗酸菌 表面抗原의 相關關係에 관한 연구

대한결핵협회 결핵연구원

김상재 · 배길한 · 이승호 · 홍영표

서울대학교병원 중앙연구실

권 혁 한

石炭酸 처리로 사멸시킨 抗酸菌과 이 균으로 感作시킨 家兔免疫血清을 이용, 酵素結合免疫分析法(ELISA)에 의해 抗酸菌 菌體表面抗原間의 상관관계를 관찰한 結果를 보면 다음과 같다.

Mycobacterium fortuitum 抗血清을 다른 菌種으로 吸着시킨 결과, *M. fortuitum*에 대한 반응은 22.8~30.2%나 감소되었고, 그와 같은 交叉反應抗體가 거의 모두 또다른 菌種과도 반응하는 것으로 보아 이들과 반응하는 抗原이 시험된 모든 遲延發育抗酸菌의 표면에도 있음을 알 수 있었다. 그러나 이와 같은 共有抗原이 지연발육균의 표면에는 그리 많지 않았다(7%미만). *M. intracellulare*는 예외로 *fortuitum*과 반응하는 항체의 결합장소를 많이 가지고 있었는데(15.6%), 그러한 항체가 인형결핵균(*M. tuberculosis*)과는 반응하지 않는 것으로 보아 또다른 교차반응항원이 추가로 존재함을 암시해 주고 있다.

지연발육균 사이의 교차반응항원은 菌種에 따라 차이가 많았지만, 상당한 양이 있음을 관찰할 수 있었고, 항원에 따라서는 매우 한정된 菌種에서만 볼 수 있었다. 특히 *M. avium-intracellulare-scrofulaceum*(MAIS)群의 균들은 항혈청의 同族反應이 그들 사이의 흡착으로 가장 크게 감소되므로 항원적 상관관계가 매우 가까움을 알 수 있었다. *M. avium*에 의해 *M. intracellulare* 항혈청의 동족반응이 76.4%나 감소되어 후자의 菌표면에 전자의 菌과 공유하는 항원이 많음을 알 수 있었는데, 특히 많은 양이 두 菌種에서만 볼 수 있는 것이고 그 다음으로는 MAIS 공유항원이었다. *M. avium* 항혈청의 동족반응은 다른 菌種로 흡착시켜도 크게 감소되지 않아 菌표면에 교차반응항원이 많지 않은 것으로 보이는데, 그 가운데서도 역시 *intracellulare*와 가장 많이 공유되어 있었고 그 다음이 *scrofulaceum*이었다. *M. scrofulaceum* 항혈청도 *avium-intracellulare*로 흡착시에 가장 크게 감소(31.8~34.5%)되었다. MAIS 菌 내에서는 *avium*과 *intracellulare* 사이가 *scrofulaceum*과 다른 두 菌種 사이보다 더 가까웠다.

抗結核菌血清의 동족반응은 *kansasii*에 의해 24.2%, *intracellulare*에 의해 19.6%, *avium*에 의

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**본 연구에 이용된 일부 菌주를 분양해 주신 중앙대학교 의과대학의 최철순 교수와 일본 Shimane 의과대학의 Hajime Saito 교수께 감사드립니다.

해 18.5%, *scrofulaceum*에 의해 15.1%, 그리고 *fortuitum*에 의해 6.7%가 감소했다. 교차반응항체의 2차 교차반응양상을 분석한 결과, 결핵균표면항원 중에는 2~3개의 지연발육균종에 국한된 것도 있었지만, 상당한 양(약 11%)은 모든 지연발육균에 공유된 항원이었고 모든 항산균에 공유된 항원도 상당히 많이 관찰되었다(약 6%).

Introduction

The recent development of mycobacterial taxonomy has been largely relied on the computer assisted numerical analysis of the various biological and biochemical characteristics of the isolates(1~3). This, however, does not solve all the problems encountered to the classification and identification of mycobacterial isolates. The precise phylogenetic relationships of mycobacteria can be elucidated if there is a technique by which the homology of the whole bacterial genomes can be tested. Unfortunately the practical application is not possible for the whole bacterial genomes but only to the certain DNA fragments, which, nevertheless, seem to be very useful to confirm hypothetical phylogeny of the phenetic clusterings based on the various morphological and biochemical characteristics(3~6). In addition to this, the various immunological procedures have also been used to investigate the phenetic differences of the species, subspecies, biobars or serobars, and in some cases they permit the better elucidation of genetic makeups of the organisms than the conventional biochemical or biological tests. The techniques based on the immunoprecipitation in agar gel have been found very useful to the serotaxonomy of mycobacteria(7~13). But the standardized antigens and antisera are not available, thus each laboratory use its own reference reagents. Schaeffer's seroagglutination technique has also opened a way of mycobacterial serotaxonomy, but the drawbacks exist in this technique too because it is applicable only to the strains forming smooth colonies(13~15). Magnusson(16) used sensitin testing to draw up a taxonomic scheme of mycobacteria although it is cumbersome and expensive procedure.

In this paper we have described an enzyme-

linked immunosorbent assay(ELISA) using the phenol-killed whole cells and their rabbit antisera with or without homologous or heterologous absorption, by which cell surface antigenic relationships among mycobacteria can be investigated regardless of whatever colonial morphology do the test strains have.

Materials and methods

1. Mycobacteria

Mycobacterium tuberculosis(H 37 Rv), *M. kansasii*(KIT 20002), *M. scrofulaceum*(KIT 30003), *M. avium*(KIT 40005, serobar 3), *M. intracellulare*(KIT 41003, serobar 4), and *M. fortuitum*(KIT 60002) were cultured as a surface pellicle on Sauton's broth medium for 3~5 weeks at 37°C. The bacillary mass was harvested by filtration and killed by treating in 2% phenol for 2 days at 37°C. The phenol killed bacilli were homogenized in a tissue homogenizer and washed with 0.067 M phosphate buffered saline(PBS, pH 7.0) five times. The bacillary suspensions so prepared were used to immunize animals and to coat the plates for ELISA.

2. Immunization of animals

The rabbits weighing 3~4 kg were immunized to one of six species mentioned above. Immunization was performed by injecting subcutaneously 20mg of killed bacillary homogenate in a Freund's incomplete adjuvant in the inguinal areas and after 4 weeks the booster immunization followed as four injections were given at two weeks interval. Animals were bled two weeks after final injection. Three rabbits were used for the immunization of each strain of mycobacteria.

The most of animals immunized with phenol-killed bacilli produced a highest level of antibody

after 4~6 injections on both ELISA and immunodiffusion tests. Immunodiffusion tests were performed using the homologous crude mixture of soluble antigens derived from the sonicated cell homogenates.

3. Enzyme-linked immunosorbent assay

The optimum concentrations of bacilli for coating the polystyrene plates and for the homologous and heterologous absorptions of antiserum have been determined prior to the main assays using the varying antiserum dilutions. It was found that 1 mg/ml of bacilli for coating the plates, 50 mg/ml of bacilli for absorption, and 1 : 500 dilution of antiserum were most suitable for the assays of antibodies to the cell surface antigens of mycobacteria in this study. The phenol-killed bacillary suspension (1 mg/ml) in a carbonate buffer (pH 9.6) was adsorbed on the microtiter plate (Flow Laboratories Inc., McLean, VA 22101, USA) overnight at 4°C and then the plates were washed and reacted with unabsorbed or homologously or heterologously absorbed antiserum diluted to 1 : 500 for 1.5 hours at 37°C. Antiserum absorptions have been undertaken by reacting serum dilution (1 : 500) containing 50 mg/ml of bacilli for 2 hours on a shaker and then overnight at 4°C. The plates were washed again with PBS-tween 80 and reacted with 1 : 600 dilution of goat antirabbit IgG peroxidase conjugated IgG fraction (Cappel Laboratories, Cochranville, PA 19330, USA). Enzyme substrates were added after washing and the reactions were stopped with 4 N sulfuric acid. Optical densities (OD) of the reactants were read at 492 nm using a Titertek Multiskan spectrophotometer (Flow Laboratories Inc.).

4. Analysis of the results

Mean OD of 5 wells was used to the analysis. The primary cross reactive antibodies (PCRA) present in the antisera were calculated according to the above formula. In a word the percentages of reduced mean ODs of the homologous reactions by the heterologous absorptions have been expressed as PCRA.

The secondary cross reactive antibodies (SCRA) represent the cross reacting antibodies secondarily reacting with the another heterologous microorganisms. They were also calculated according to the above formula.

Results

A considerable amount of the cross reacting antibodies were detected in all mycobacterial antisera as ODs of the heterologous reactions were nearly same with ODs of the homologous reactions if the sera were not absorbed with homologous or heterologous microorganisms. However, most of the cross reacting antibodies could be absorbed out by either homologous or heterologous microorganisms, while the large amount of homologous antibodies remained even after heterologous absorption. In general, the amounts of the primary cross reacting antibodies (PCRA), which could be absorbed out by heterologous organisms in homologous system, were relatively less in *M. kansasii* and *M. avium* antisera than in other antisera even if the great differences existed depending on the antigenic relationships between the strains studied, as seen in Table 1~7.

The slowly growing mycobacteria usually absorbed out more amount of nonspecific antibodies

$$PCRA = \frac{\frac{OD \text{ of unabsorbed homologous serum} - OD \text{ of homologous serum absorbed with heterologous bacilli}}{OD \text{ of unabsorbed homologous serum} - OD \text{ of homologous serum absorbed with homologous bacilli}}}{\frac{OD \text{ of unabsorbed homologous serum} - OD \text{ of homologous serum absorbed with heterologous bacilli}}{OD \text{ of unabsorbed homologous serum} - OD \text{ of homologous serum absorbed with homologous bacilli}}} \times 100$$

$$SCRA = \frac{\frac{OD \text{ of unabsorbed serum to the 1st heterologous bacilli} - OD \text{ of serum absorbed with the 2nd heterologous bacilli, to the 1st heterologous bacilli}}{OD \text{ of unabsorbed serum to the 1st heterologous bacilli} - OD \text{ of serum absorbed with the 1st heterologous bacilli}}}{\frac{OD \text{ of unabsorbed serum to the 1st heterologous bacilli} - OD \text{ of serum absorbed with the 2nd heterologous bacilli, to the 1st heterologous bacilli}}{OD \text{ of unabsorbed serum to the 1st heterologous bacilli} - OD \text{ of serum absorbed with the 1st heterologous bacilli}}} \times 100$$

in the *M. fortuitum* antiserum when it was compared with the *M. fortuitum* absorbed out nonspecific antibodies in the antisera of the slow growers except *M. intracellulare*. Almost all of those cross reacting antibodies between *M. fortuitum* and the slow growers also cross reacted secondarily to the other heterologous species, indicating they have been produced by the cell surface antigens shared by all the mycobacteria used in this study. *M. intracellulare*, however, produced some additional *fortuitum* absorbable antibodies, which cross reacted to all but *M. tuberculosis*.

Although *M. avium* behaved somewhat unusually, there were evidently close antigenic relationships among *M. avium-intracellulare-scrofulaceum*(MA-IS) complex. *M. intracellulare* produced a huge amount of *avium* absorbable antibodies, but *M. avium* did not even if it produced more *intracellulare* absorbable antibodies than the other nonspecific antibodies. The largest heterologous absorption of *M. scrofulaceum* antiserum was also found with *M. avium-intracellulare*. The antigenic relationship, however, seemed to be closer between *M. avium* and *intracellulare* than between *M. scrofulaceum* and either *avium* or *intracellulare*.

Anti-*M. tuberculosis* serum showed a mean OD of 1.775 in the homologous system, but when the serum was absorbed with *M. tuberculosis*, *kansasii*, *intracellulare*, *avium*, *scrofulaceum* and *fortuitum*, it decreased to 0.134, 1.376, 1.454, 1.472, 1.527, and 1.665 respectively (Table 2). Thus the calculated PCRA to *M. kansasii* in the homologous system was 24.3% and 19.6% to *intracellulare*, 18.5% to *avium*, 15.1% to *scrofulaceum*, and 6.7% to *fortuitum* as seen in Table 1. The OPs of AMTB to *M. kansasii*, *scrofulaceum*, *avium*, *intracellulare*, and *fortuitum* were 1.663, 1.805, 1.828, 1.857, and 1.853 respectively, but the homologous or heterologous absorption decreased these values to a great extent except *M. fortuitum* absorption, which did not remove the secondary cross reacting antibodies (SCRAs). Almost all of antibodies reacting to *M. fortuitum* in *M. tuberculosis* antiserum, however, were absorbed out not only

by homologous but also by heterologous organisms, indicating that the antigens shared by *M. fortuitum* on human tubercle bacilli were shared by all the mycobacteria of this study. The large amount of antibodies have also been produced by *M. tuberculosis* surface antigens shared by all the slow growers but not by *M. fortuitum*, as the PCRA to the slow growers could have been absorbed out to a great extent by the second heterologous slowly growing mycobacteria. And some of those cross reacting antibodies to the slow growers in the unabsorbed *M. tuberculosis* antiserum reacted not to all but only to one or two second slow growers, indicating the presence of the shared antigens limited to one or few slow growers. For example, only 79% (SCRA) of *M. tuberculosis* PCRA to *M. kansasii* were absorbed out by *M. scrofulaceum*, but much more by the other slow growers (Table 1). The cross reacting antibodies to *M. avium* was also absorbed out nearly all by *M. intracellulare* but only 86~89% by *M. kansasii* or *scrofulaceum*.

The homologous reaction of the antiserum obtained from the *M. kansasii* immunized animals was not decreased much by the heterologous absorptions when it was compared with *M. tuberculosis* antiserum as seen in Table 1~3. Whether this result indicates the paucity of shared antigens on *M. kansasii* cell surfaces is not known. In the Table 3, the reactivity of *M. kansasii* antiserum to the other species before or after homologous or heterologous absorptions, were summarized. Here again, *M. kansasii* antigens shared by *M. fortuitum* were apparently shared by all the mycobacteria studied. The slow growers, of course, absorbed out much more *M. kansasii* antibodies than *M. fortuitum* and most of those antibodies confined to the slow growers might have been produced by the antigens shared by all the slow growers studied.

The *M. scrofulaceum* antiserum showed a different reactivity to the slow growers from that of the *M. tuberculosis* or *kansasii* antiserum, but it showed the same reactivity pattern to *M. fortui-*

Table 1. The Primary and Secondary Cross Reactions of Rabbits Anti-mycobacterial Sera

Antiserum	Antiserum absorbed bacilli	Plate coated bacilli					
		MTB	MKS	MSF	MAU	MIC	MFT
AMTB	MKS	(24.3)	100.0	94.6	86.4	86.1	99.6
	MSF	(15.1)	78.8	100.0	88.7	88.2	90.6
	MAU	(18.5)	88.2	80.1	100.0	96.4	98.4
	MIC	(19.6)	93.6	81.4	99.0	100.0	100.0
	MFT	(6.7)	33.9	16.3	22.5	17.0	100.0
AMKS	MTB	100.0	(4.7)	96.2	100.0	88.4	100.0
	MSF	100.0	(5.8)	100.0	100.0	97.6	100.0
	MAU	93.7	(5.7)	92.3	100.0	99.1	98.6
	MIC	100.0	(7.7)	98.0	100.0	100.0	100.0
	MET	46.8	(4.0)	34.7	37.8	33.4	100.0
AMSF	MTB	100.0	100.0	(15.0)	15.2	17.0	95.5
	MKS	89.2	100.0	(17.5)	19.1	24.1	98.1
	MAU	83.3	90.0	(31.8)	100.0	95.4	98.1
	MIC	90.6	98.8	(34.5)	99.3	100.0	100.0
	MFT	25.3	41.6	(6.1)	13.4	12.8	100.0
AMAU	MTB	100.0	98.9	38.0	(5.1)	12.5	100.0
	MKS	98.6	100.0	48.3	(4.8)	14.0	100.0
	MSF	100.0	97.8	100.0	(5.0)	100.0	99.1
	MIC	100.0	100.0	100.0	(9.3)	100.0	100.0
	MFT	9.6	29.4	8.8	(3.6)	3.3	100.0
AMIC	MTB	100.0	100.0	21.4	26.4	(8.2)	18.2
	MKS	83.4	100.0	58.2	41.5	(18.0)	82.4
	MSF	99.0	100.0	100.0	59.5	(23.5)	99.3
	MAU	96.2	95.2	92.7	100.0	(76.4)	94.0
	MFT	53.0	70.4	38.0	31.9	(15.6)	100.0
AMFT	MTB	100.0	100.0	98.1	88.9	92.3	(26.2)
	MKS	100.0	100.0	95.6	92.5	98.7	(26.9)
	MSF	96.2	95.1	100.0	91.7	100.0	(30.2)
	MAU	100.0	100.0	100.0	100.0	100.0	(29.3)
	MIC	93.1	87.8	98.3	84.7	100.0	(22.8)

*MTB=*M. tuberculosis*, MKS=*M. kansasii*, MSF=*M. scrofulaceum* MAU=*M. avium*.

MIC=*M. intracellulare*, MFT=*M. fortuitum*, AMTB=MTB antiserum, AMKS=MKS antiserum,

AMSF=MSF antiserum, AMAU=MAU antiserum, AMIC=MIC antiserum, AMFT=MFT antiserum.

**The numbers indicate per cent.

***The numbers in parentheses are percentages of the primary cross reacting antibodies in the homologous system.

as seen in tables 1~4. The homologous reaction(mean OD=1,590) of *M. scrofulaceum* antiserum was decreased to a greatest extent by the *M. avium* and *intracellulare* absorptions, indica-

ting a close antigenic relationships between the members of MAIS complex. The heterologous absorptions of the cross reacting antibodies in order to measure SCRA disclosed that large am-

Table 2. Reactivity of Anti-*M. Tuberculosis*(AMTB) Serum to the Homologous and Heterologous Mycobacteria

Plate coated bacilli	Unabsorbed serum	Antiserum absorbed bacilli					
		MTB	MKS	MSF	MAU	MIC	MFT
<i>Tuberculosis</i>	1.775	0.134	1.376	1.527	1.472	1.454	1.665
<i>Kansasii</i>	1.663	0.130	0.236	0.538	0.404	0.327	1.179
<i>Scrofulaceum</i>	1.805	0.135	0.391	0.310	0.607	0.588	1.562
<i>Avium</i>	1.828	0.135	0.386	0.347	0.159	0.175	1.452
<i>Intracellulare</i>	1.857	0.145	0.414	0.380	0.242	0.182	1.573
<i>Fortuitum</i>	1.853	0.108	0.162	0.315	0.183	0.139	0.156

For the explanation of the abbreviations, see Table 1.

The numbers represent the mean optical densities(ODs) of the reactants.

ount of antibodies reacting to the *M. avium* or *intracellulare* could have been absorbed out most by MAIS and much less by the other slow growers and least by *M. fortuitum*. This finding permits to figure out the antigenic structure of *M. scrofulaceum* cell surface on which most of the shared antigens were of the MAIS complex and a significant amount shared by all the slow growers and a small amount shared by all the mycobacteria.

Antiserum of *M. avium* immunized animals showed relatively small level of decrease in the homologous reaction by the heterologous absorptions as it was in *M. kansasii* antiserum(see Tables 1,3 and 5). However, *M. intracellulare* absorbed out more antibodies(9.3%) than the other species and all of those *M. intracellulare* absorbable antibodies in *M. avium* antiserum were also removed by *M. scrofulaceum* absorption. Thus, although *M. avium* did not produce much of the cross reacting antibodies, it certainly induced production of more antibodies against the MAIS complex shared antigens. For example, mean ODs of the heterologous reactions to *M. intracellulare* or *scrofulaceum* were not decreased much by *M. tuberculosis* or *kansasii* absorption but decreased a lot by the MAIS complex(see Table 5).

The homologous reaction of anti-*M. intracellulare* serum was decreased to a great extent(76.4 %) by *M. avium* absorption, indicating a large

antigenic sharing with *M. avium* even though the reverse was not the case(Tables 1 and 6). A considerable amount of antibodies reacting to *M. avium*, however, did not react to the other species, suggesting they have been produced by shared antigens only between *M. avium* and *intracellulare*. Analysis of SCRA revealed that there were of course antigens shared only between MAIS complex and between all the slow growers. *M. intracellulare* antibodies reacting to *M. fortuitum* behaved differently because they were not absorbed out much by *M. tuberculosis* as seen in Table 6. These antibodies, which had a very few SCRA to *M. tuberculosis* but much more to the other species, might have been produced by the antigens shared by *M. fortuitum* but somewhat different from *fortuitum* shared antigens on the other species.

Approximately 30.2% of the homologous reaction of *M. fortuitum* antiserum has been reduced by *M. intracellulare* absorption and 22.8% by *M. avium* as seen in tables 1 and 7. Antibodies reacting to the latter species cross reacted the nearly 100%(SCRA) to all the other slow growers, however, there seemed to be a very small amount of additional antibodies, which showed a secondary cross reaction to a few limited species. Mean OD of unabsorbed anti-*M. fortuitum* serum to *M. intracellulare* cells was 1.772 and it decreased to 0.141 and 0.228 by *M. scrofulaceum* and *tuberculosis* but to 0.316 and 0.367 by *M. kansasii* and

Table 3. Reactivity of Anti-*M. Kansasii*(AMKS) Serum to the Homolous and Heterologous Mycobacteria

Plate coated bacilli	Unabsorbed serum	Antiserum absorbed bacilli					
		MTB	MKS	MSF	MAU	MIC	MFT
<i>Tuberculosis</i>	1.518	0.065	0.179	0.180	0.263	0.178	0.891
<i>Kansasii</i>	1.742	1.672	0.254	1.656	1.657	1.628	1.683
<i>Scrofulaceum</i>	1.522	0.222	0.170	0.126	0.273	0.197	1.053
<i>Avium</i>	1.571	0.106	0.152	0.115	0.106	0.110	1.034
<i>Intracellulare</i>	1.659	0.359	0.188	0.224	0.201	0.133	1.164
<i>Fortuitum</i>	1.580	0.090	0.102	0.101	0.122	0.082	0.066

For the explanation of the abbreviations, see Table 1.
The numbers represent the mean ODs of the reactants.

Table 4. Reactivity of Anti-*M. Scrofulaceum*(AMSF) Serum to the Homologous and Heterologous Mycobacteria

Plate coated bacilli	Unabsorbed serum	Antiserum absorbed bacilli					
		MTB	MKS	KSF	MAU	MIC	MFT
<i>Tuberculosis</i>	1.630	0.104	0.297	0.136	0.385	0.277	1.252
<i>Kansasii</i>	1.253	0.165	0.166	0.136	0.275	0.179	0.801
<i>Scrofulaceum</i>	1.590	1.375	1.338	0.152	1.132	1.094	1.502
<i>Avium</i>	1.682	1.453	1.394	0.175	0.142	0.185	1.480
<i>Intracellulare</i>	1.695	1.437	1.329	0.178	0.249	0.177	0.501
<i>Fortuitum</i>	1.498	0.161	0.124	0.098	0.125	0.086	0.094

For the explanation of the abbreviation, see Table 1.
The numbers represent the mean ODs of the reactants.

avium, thus the SCRAs to the former two species were 98.3% and 93.1% but to the latter two species, 87.8% and 84.7%. From this observation, it was clear that the nearly one fourth of mycobacterial cell surface antibodies in the *M. fortuitum* antiserum might have been produced by the antigens shared by all the mycobacteria studied and a small additional amount by the antigens shared by a few limited species.

Discussion

It has long been understood that there are considerable amount of the antigenic cellular constituents or metabolites shared by the different species of mycobacteria and that such different antigenic structures are closely related to the their phylogeny. No one has doubt about that configu-

ration of the mycobacterial antigens may reflect far more genetic make-up of the organisms than the ordinary biological or biochemical tests. Unfortunately the well standardized methods permitting to figure out the antigenic shape of mycobacteria are not available yet in spite of a recent dazzling development of the immunological technology. Schaeffer's seroagglutination tests opened a way of serotaxonomic scheme of mycobacteria and it has been found very useful for the taxonomy of MAIS complex and some other mycobacteria(13~15). However this technique is not applicable to the mycobacterial species or strains whose colonies are rough or dry. Immunodiffusion and immunoelectrophoresis have also been found useful to investigate the antigenic relationships of mycobacteria although they have some drawb-

Table 5. Reactivity of Anti-*M. Avium*(AMAU) Serum to the Homologous and Heterologous Mycobacteria

Plate coated bacilli	Unabsorbed serum	Antiserum absorbed bacilli					
		MTB	MKS	MSF	MAU	MIC	MFT
<i>Tuberculosis</i>	1.684	0.045	0.185	0.156	0.164	0.156	1.538
<i>Kansasii</i>	1.299	0.160	0.145	0.172	0.147	0.146	0.960
<i>Scrofulaceum</i>	1.710	1.113	0.951	0.140	0.123	0.118	1.572
<i>Avium</i>	1.901	1.810	1.817	1.812	0.134	1.736	1.838
<i>Intracellulare</i>	1.801	1.595	1.570	0.133	0.144	1.148	1.572
<i>Fortuitum</i>	1.503	0.079	0.099	0.135	0.122	0.087	0.087

The explanation for the abbreviations, see Table 1.

The numbers represent the mean ODs of the reactants.

Table 6. Reactivity of Anti-*M. Intracellulare*(AMIC) Serum to the Homologous and Heterologous Mycobacteria

Plate coated bacilli	Unabsorbed serum	Antiserum absorbed bacilli					
		MTB	MKS	MSF	MAU	MIC	MFT
<i>Tuberculosis</i>	1.676	0.083	0.369	0.123	0.168	0.108	0.845
<i>Kansasii</i>	1.423	0.133	0.140	0.123	0.202	0.097	0.520
<i>Scrofulaceum</i>	1.672	1.341	0.774	0.128	0.241	0.108	1.086
<i>Avium</i>	1.736	1.314	1.073	0.785	0.134	0.138	1.226
<i>Intracellulare</i>	1.764	1.633	1.478	1.390	0.549	0.174	1.516
<i>Fortuitum</i>	1.698	1.411	0.397	0.131	0.214	0.105	0.120

For the explanation of the abbreviations, see Table 6.

The numbers represent the mean ODs of the reactants.

acks too because the antigens and antisera used to these procedures are not standardized so that each laboratory uses its own reference reagents. The techniques related to the immunoelectrophoresis are somewhat cumbersome to perform. Stanford et al(12, 13) classified the soluble cell extract antigens of mycobacteria into the four groups by immunodiffusion tests in which group i comprise the broadly cross reacting antigens and group ii, the antigens limited to the slow growers, group iii, the antigens limited to the rapid growers, and group iv, the species specific antigens. Chaparas(7) calculated the cross reactivity between the several species of mycobacteria by immunoelectrophoresis and he found that *M. fortuitum* shared 76% of their soluble antigens with *M. smegmatis* and 64% with *M. chelonae*, 60%

with *M. tuberculosis*, 44% with *M. kansasii*, and 40% with *M. gordonae*.

In this study we have used the enzyme-linked immunosorbent assay in the investigation of antigenic relationships among mycobacteria. Although the amount of antibodies to the different antigenic determinants, detected by ELISA, does not necessarily mean the equal amount of antigens present in the reactants probably due to the unequal immunogenicity of each antigenic components, ELISA, however, permits the quantitative measurements of the specific and cross reactive antibodies between the species on which the antigenic relationships between the species, subspecies, and serobars, could be elucidated with relative accuracy. In order to overcome the variations in antigen preparations, the phenol-killed wh-

Table 7. Reactivity of Anti-*M. Fortuitum*(AMFT) Serum to the Homologous and Heterologous Mycobacteria

Plate coated bacilli	Unabsorbed serum	Antiserum absorbed bacilli					
		MTB	MKS	MSF	MAU	MIC	MFT
<i>Tuberculosis</i>	1.693	0.028	0.112	0.180	0.323	0.239	0.118
<i>Kansasii</i>	1.506	0.166	0.103	0.227	0.269	0.185	0.168
<i>Scrofulaceum</i>	1.698	0.187	0.204	0.088	0.258	0.115	0.127
<i>Avium</i>	1.760	0.085	0.083	0.094	0.074	0.081	0.108
<i>Intracellulare</i>	1.772	0.228	0.316	0.141	0.367	0.054	0.113
<i>Fortuitum</i>	1.873	1.410	1.399	1.356	1.471	1.340	0.109

For the explanation of the abbreviations, see Table 1.
The numbers represent the mean ODs of the reactants.

ole cells were used instead of the soluble antigens in this study.

The overall findings showed that the immunized animals produced a large amount of species specific antibodies and a significant amount of the nonspecific cross reacting antibodies as well, suggesting that there are large amount of species specific antigens on the cell surface along with a considerable amount of shared antigens. *M. kansasii* and *avium* induced large amounts of specific and nonspecific antibodies in the animals, but they apparently did not contain much of shared antigens on the cell surface because their homologous reactions were not decreased much by the heterologous absorptions.

The homologous reaction of *M. tuberculosis* antiserum was decreased 24.3% by *M. kansasii* absorption, indicating that the corresponding amount of antibody binding sites on *M. tuberculosis* cell surface were shared by the absorbed organisms. Those cross reactive antibodies also reacted to the other heterologous mycobacteria, for example, 33.9% of them reacted with *M. fortuitum*, and 78.8% with *M. scrofulaceum*, 88.2% with *M. avium* and 93.6% with *M. intracellulare*. Therefore the cross reacting antibodies in anti-*M. tuberculosis* serum might have been produced by somewhat broadly shared antigens, which might be proportional to the percentages of SCRA's. On the premise that the amount of antibodies abso-

rbed out by the heterologous organisms are equal to the amount of binding sites present on the homologous bacillary surfaces, less than 7% of antibody binding sites on the tubercle bacilli were shared by *M. fortuitum* and approximately 10~14 % by the other slow growers. If added one or two species shared sites, more than one fourth of antibody binding sites on the tubercle bacilli were shared by the other species. However two or three species could remove all the cross reacting antibodies and thus make it possible to produce the species specific antiserum. In spite of that the tubercle bacilli induced production of a considerable amount of antibodies against *M. kansasii*, *M. kansasii* immunized animals, however, did not produce much of antibodies reacting with tubercle bacilli.

Approximately 23~30% of the antibody binding sites of *M. fortuitum* cell surfaces were found shared mostly by all the slow growers studied. On the contrary, the sera of the animals immunized with the slowly growing mycobacteria could not be absorbed out much of their homologous reactions by *M. fortuitum* cells. *M. intracellulare*, however, possessed exceptionally large amount of *M. fortuitum* reacting antibodies(15.6%) of which most did not react with *M. tuberculosis*, suggesting that they might have been produced by the *fortuitum* shared antigens on *M. intracellulare* cell surfaces, which are different from the *fortu-*

itum shared antigens on the other slow growers.

M. intracellulare has been found containing a large amount of surface antigens shared by *M. avium* only and by the MAIS complex because 76.4% of the homologous reaction of anti-*M. intracellulare* serum was absorbed out by *M. avium* and 23.5% by *M. scrofulaceum*. But the homologous reaction of *M. avium* antiserum was not absorbed out much by the other mycobacteria of which, of course, *M. intracellulare* cross reacted most. *M. scrofulaceum* antiserum was also absorbed out to a largest extent(32~35%) by *M. avium-intracellulare*, indicating a close antigenic relationships between the members of MAIS complex. Within the MAIS complex, antigenic relationship between *M. avium* and *intracellulare* seemed to be closer than between *M. scrofulaceum* and either one of the others.

In general the antigenic relationships of mycobacteria observed by the various serological procedures using the polyvalent soluble extracts seemed to show less remarkable differences between species than those observed by ELISA using the whole cells.

Summary

The enzyme-linked immunosorbent assay using the phenol-killed bacilli and their rabbits antisera has been used to the investigation of the cell surface antigenic relationships among mycobacteria.

In the homologous reaction of anti-*Mycobacterium fortuitum* serum, approximately 22.8 to 30.2 %(PCRAs) of the reaction could have been inhibited by the heterologous absorptions and almost all of those antibodies cross reacted with the second heterologous mycobacteria, suggesting that the shared antigenic determinants on the *M. fortuitum* cell surfaces are common to all the slowly growing mycobacteria studied. On the contrary, those common antigens were present much less (<7%) on the slow growers except *M. intracellulare*, which seemed to possess additional *fortuitum* shared antigens common to all but *M. tuberculosis*.

A considerably large amounts of the cell surface antigens have been shared by all the slow growers and some, of course, by a few limited species. The *M. avium-intracellulare-scrofulaceum* (MAIS) complex showed a closer antigenic relationship as a largest decrease of the homologous reaction has always been observed by the heterologous absorptions between them. *M. avium* inhibited 76.4% of antibody binding on the *M. intracellulare* cell surfaces in the homologous system, of which the large amount of antibody binding sites were common to only these two species and also a significant amount, to the all members of MAIS complex. The reaction of *M. avium* antiserum, however, was not reduced much by the heterologous absorptions even though a largest amount of PCRAs, still could be absorbed out by *M. intracellulare* and next by *M. scrofulaceum*. Absorption of *M. scrofulaceum* antiserum by *M. avium-intracellulare* also reduced the homologous reaction to a greatest extent(31.8~34.5%). Thus it was apparent that *M. avium-intracellulare* are antigenically closer than between either one of them and *M. scrofulaceum*.

The PCRAs of anti-*M. tuberculosis* serum were 24.3% to *kansasii*, 19.6% to *intracellulare*, 18.5% to *avium*, 15.1% to *scrofulaceum*, and 6.7% to *fortuitum* and the SCRA of them indicated that in addition to the some antibody binding sites limited to 2~3 slow growers there were large amount of antigenic determinants(approximately 14%) shared by only slow growers and also a considerable amount by all mycobacteria studied.

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