STUDIES ON CELL-MEDIATED IMMUNE RESPONSE TO TREPONEMAL ANTIGENS

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by

Olive Cheu Moi Cheung

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"STUDIES ON CELL-MEDIATED IMMUNE RESPONSE TO TREPONEMAL ANTIGENS"

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OLIVE CHEU MOI CHEUNG

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Seven strains of treponemes (Treponema scoliodontum, T. phagedenis biotype English Reiter, T. phagedenis biotype Kazan 5, T. phagedenis biotype Kazan 4, T. denticola, T. refringens biotype Nichols, and T. refringens biotype refringens), members in the order of spirochaetales, were used for the studies on cell-mediated immune response to treponemal antigens.

Cytoplasmic antigens of treponemes were capable of eliciting cellular immune reactions in the sensitized guinea pigs in terms of delayed hypersensitivity (skin test) and inhibition of migration of macrophage (macrophage migration inhibition test).

The results of macrophage migration inhibition tests did not show strongly correlation with those of skin tests in both homologous and heterologous antigenic groups.

Using the program of mixed factorial design, it was found that by skin test and macrophage migration inhibition, the degree of sensitization among strains was different. The same variation was also observed in the degree of reaction in the presence of different treponemal antigens.

The mutual relationships among treponemes were estimated by the Duncan's New Multiple-Range test and a simple percentage calculation. Two groups were found by skin tests while one group was found by macrophage migration inhibition test. The conclusion of the antigenic mutual relationships depends on the period of sensitization.

The partially purified Treponema pallidum failed to induce skin reactions on rabbits infected with live Treponema pallidum which has been

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INTRODUCTION

Evidence was shown that infection with Treponema pallidum stimulates a cell-mediated immune response in the host in certain stages of infection but not in others. The presence of specific (T. pallidum immobilizing antibody and fluorescent antibody after treponemal absorption) was nonspecific VDRL (Veneral Disease Research Laboratory) antibodies play no role or a very limited role in resistance to reinfection[1]. In 1950, Csonka discovered that cutaneous delayed hypersensitivity to treponemal antigens was associated with latent syphilis[2]. A local reaction, analogous to the tuberculin test, occurred at this latent stage when T. pallidum antigen was injected intradermally into the subject. Fulford (1972) reported on an in vitro reaction, the leukocyte migration inhibition reaction to treponemal antigens. He was able to demonstrate that migration of leukocytes from patients with primary syphilis was stimulated by Reiter Treponemal protein antigens while the migration of leukocytes obtained from late active syphilis was inhibited. It was also shown that no stimulation or inhibition of migration occurs in secondary syphilis[3]. Activation of macrophages is a central feature of cell-mediated immunity. After infection, macrophage possesses enhanced phagocytic and bactericidal properties, not only against the infecting organism but also against antigenically unrelated ones[4]. The latter phenomenon was demonstrated by Schell and Musher (1974) by infecting rabbits with T. pallidum and the animal's ability to resist subsequent challenge of Listeria monocytogenes was enhanced between three and five weeks after infection, coincident the onset and regression of the generalized syphilitic eruption[5]. Musker

et al.(1973)[6] investigated in vitro reaction of syphilitic patients' lymphocytes to Treponema refringens by using lymphocyte transformation assay. Those investigators found that T. refringens stimulated lymphocytes from normal subjects to undergo blastic transformation in vitro, in contrast to the significantly lesser extent of transformation of lymphocytes from patients with primary and secondary syphilis. However, lymphocytes from pirmary or secondary syphilitic patients who had been treated by antibiotics for six to ten weeks was increased to normal level when exposed to T. refringens. It was therefore suggested that lymphocyte response to T. refringens in vitro is an indication of the existence of cell-mediated immunity (CMI). Primary and secondary syphilitic patients are regarded as having defective cell-mediated immunity because of the low lymphocyte transformation response to T. refringens.

All these observations suggest that cell-mediated immunity is involved in the clinical evolution of syphilis. Cell-mediated immunity is suppressed in the early stages of infection and becomes active at the time that latency is induced. Progression of active syphilis might result from the failure to stimulate cell-mediated immunity response or from suppression of cell-mediated immunity response.

This investigation has been set to answer two questions: 1) do the cytoplasmic antigens of treponemes elicit cell-mediated immunity, and 2) what is the range of immunological specificity of the antigens evoking such response. In these studies, the nature of treponemal antigens and their mutual relationships, and particularly those relevent to Treponema pallidum and syphilis would thus be further elucidated.

Background of immunological field, the cell-mediated immunity

Immunity can be divided into two classes, namely, cell-mediated immunity (CMI) and humoral immunity.

Humoral immunity can be passively transferred from an immune organism to nonimmune one by serum. As to cell mediated immunity which cannot be passively transferred by serum, the lymphoid cells are the mediators of the immune reactions[4].

An *in vitro* test (delayed hypersensitivity skin test) and an *in vitro* test (macrophage migration inhibition test) were used in the studies of treponemal cell-mediated immunity.

The histopathology of delayed hypersensitivity and inflammatory reactions have shown that similar cell types are involved (Cruickshank, R. (1965). In "Medical Microbiology", *loc. cit.*). Sell and Asofsky (1968)[7] characterized delayed hypersensitivity by the following criteria: a) delayed hypersensitivity can only be transferred by specifically sensitized cells. Lymphoid cells from lymph node, spleen and peripheral blood leukocytes have been demonstrated to be able to transfer cell-mediated immunity; b) the skin reaction time is longer than immediate hypersensitivity. The cellular infiltrate usually reaches a maximum at 24 hours following antigen injection, giving rise to a palpable induration, and begins to subside after 48 hours; c) the histological picture of the reaction site is different from Arthus reaction by the infiltration of mononuclear leukocytes, mostly lymphocytes and macrophages, this contrasts with the essentially polymorph character of the arthus reaction.

The technique of migration inhibition using capillary tubes was

introduced by George and Vaughan (1962)[8] and extended by David et al. (1964)[9], and it is considered to be correlated with cellular immunity. David et al (1964) found that the macrophages obtained from the tuberculin sensitized animals were inhibited from migration in the presence of specific antigen. Later, David[9], Bloom and Bennett [10, 11] reported that the capillary tube-migration inhibition assay required: 1) a macrophage cell population, usually obtained from peritoneal exudate by the injection of mineral oil into the peritoneal cavity; 2) a few specifically sensitized lymphocytes; and 3) the specific antigen. Benacerraf and Gel[12] described that the carrier protein of the heptan-protein conjugate was responsible for the specificity of delayed reaction.

A soluble factor[9] which is non-dialysable, sensitive to proteolytic enzyme such as trypsin, heat stable by incubation at 56°C for thirty minutes and having a molecular weight of 67,000 was recovered from the supernatants of sensitized lymphocytes culture after the contact of specific antigen. This factor was reported by Thor et al (1968)[13] that it could inhibit the migration of normal guinea pig peritoneal exudate cells (PEC). The factor responsible for the activity in the supernatant was cryostable and named migration inhibition factor (MIF)[9].

Although the inhibition of migration cannot be directly correlated with the diameter of skin reaction, it has been demonstrated to correlate with cellular immunity in vivo. David et al.[9] (1964) reported that a positive migration inhibition of PEC from an animal sensitized with tuberculin could be demonstrated when the animal had delayed skin reaction to the same antigen. This inhibition was specific for the immuniz-

ing antigen and occurred whether or not circulating antibody was present. However, a few derivations from the preceding correlationship between macrophage migration inhibition and delayed skin reaction have been reported such as in the case of BCG vaccinated guinea pigs, a negative skin test was accompanied by a positive migration inhibition test[14].

In conclusion, the production of MIF by lymphoid cells from a sensitized animal by incubating the sensitized lymphoid cells with the specific antigen is widely accepted as an *in vitro* indication of cell-mediated hypersensitivity. Therefore, it is a useful tool in the studies of cell-mediated immunity, i.e., allograft immunity, autoimmunity, tumor immunity, and delayed hypersensitivity in man.

REVIEW OF PERTINENT LITERATURE ON TREPONEMES

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF TREPONEMES

Treponema is a member of the order spirochaetales, and has been either isolated or observed microscopically in many animals. It can be seen in Table I[15-17] that positive isolation of treponemes were obtained from all carnivors, omnivores and most of the herbivors.[15-17] Treponemes have been isolated from the oral cavity, genital-anal areas, rumen and intestinal material from the flora of man and animal (see Table II).[18] The oral and genital-anal treponemes were isolated from the flora of men and animals. T. denticola is the most frequently isolated treponemes from the oral cavity of man. Treponema hyodysenteriae has been isolated from the intestines of swine, and Rumen fluid requiring treponemes were isolated from the intestines and rumen contents of cattle.[15-17] Spirochaetales is helically coiled, slender, flexuous, unicellular, 5 to 20 µm long and 0.09 to 0.5 µm wide; gram-negative but is best observed by dark field microscopy or phase contrast microscopy. The organisms are mobile and move in a cork-screw or serpentine fashion. Generally, the cell consists of an outer cell envelope and an inner protoplasmic cylinder. Between the cell envelope and the protoplasmic cylinder there is one of more axial fibrils inserted at each end of the cell. Some of the treponemes ferment glucose and others, amino acids. They are anaerobes and are catalase and oxidase negative. The cultivable species contain 36 to 40 mole percent guanine and cytosine in deoxyribonucleic acid (DNA).

Treponemes can be divided into two groups: those non-pathogenic but cultivable, and those pathogenic but noncultivable in vitro which include Treponema pallidum, the cause of syphilis; T. pertenus, the cause

of rabbit syphilis. The non-pathogenic ones are T. phagedenis, which comprises the Reiter treponema, English Reiter and all the Kazan strains; T. refringens, which comprises the Noguchi and avirulent cultivated Nichols strain; and T. denticola.

Attempts to grow virulent T. pallidum in tissue culture, organ culture, or in embryonating chicken eggs have not been successful [19, 20] except in only one experiment. Kast (1929) infected rabbits with the Nichols strain and he was able to cultivate the treponema from testicular chancrous lesions in hormone-ascitic fluid medium.[21] However, there was morphological change and the culture was consistently non-pathogenic in rabbits. Furthermore, the experiment cannot be repeated.

The cultivable treponemes can be grown in media and their characteristics can be studied like any other bacteria. Some characteristics and end products of fermentation of those cultivable treponemes are listed in Table III [15, 16, 22, 23] and Table IV[15, 16, 22, 23]. As shown in Table III, there is no significant difference in size among treponemes. Treponema phagedenis and T. macrodentium utilize glucose but not lactate. However, T. phagedenis produce indol, acetate while T. macrodentium produces formate, acetate, lactate and succinate as end products. T. denticola and T. scolidontum do not utilize glucose or lactate. The former produces indole, amonia, acetate and lactate as end products, on the other hand the latter gives acetate, propionate and isobutyrate as end products. T. oralis is the only treponema that utilizes lactate but not glucose, yielding indol, acetate and propionate but ammonia as end products. T. vincentii does not utilize any sugars and gives rise to indol

and acetate as end products.[15, 16, 22, 23]

The most important factor for cultivation of treponemes is fatty acid. Either short chain volatile fatty acids or long chain fatty acids are required for cultivable treponemes. Short chain acids can be provided by rumen fluid from cattle or other ruminants or by artificial mixtures. Long chain fatty acids can be supplied from animal sera. Rabbit, horse, sheep, and cattle sera have been used in culture media at a concentration of 10% to 20%. There is no single medium for the isolation of all the known species of treponemes, nor is there a general medium for the optimal growth of various species of treponemes. Media used for the cultivation of treponemes are listed in Table V.[15, 22-25]

Prior to 1959, treponemes were grown only in liquid or semisolid media. In 1959, Socransky et al., were able to grow some oral treponemes on both streaked and poured plates.[26] Cultures were grown in anaerobic conditions, and the number of colonies. Agar media used for isolation and colonial growth of treponemes are shown in Table VI.[15, 22-25,27]

In general, the colonies produced by different treponemes can be divided into two distinct types: the flat, diffuse colonies; and raised, discrete colonies. The diffuse colonies occurred entirely within the agar and appeared flat. The discrete colonies which can be further divided into rhizoid and round subtypes are raised above the agar surface and have a well-defined margin. The discrete colonies are usually much smaller than the diffuse colonies and are found only when treponemes produce more than one type of colony.[24] Hardy et al., (1963) reported that the Reiter treponema formed three distinct colony types after 6 to 7 days of anaerobic incubation. Two types of the colonies, namely rhizoid and

subsurface diffuse types, were prominant. The third type was small, round, convex, glistening and mucoid. The small oral treponemes readily grow after 6 to 7 days of incubation, and the colonies are similar in appearance with Reiter treponema. Kazan strains yield diffuse, homogenous colonies after 6 to 8 days of incubation. Noguchi and Nichols strains require the longest period for the appearance of colonies, they both require 14 days of incubation to produce diffuse and round surface colonies.[24]

TABLE I

ANIMAL TREPONEMES ISOLATED FROM OR SEEN BY MICROSCOPY (SMIBERT, 1971., HARIS, 1972., AND BRYANT, 1952)[15-17]

Animal	Present	Animal	Present
Chimpanzee	+	Raccoon	+
Squirrel monkey	+	Guinea pig	–
Owl monkey	+	Striped skunk	+
Rhesus monkey	+	Spotted skunk	+
Red fox	+	Syrian hamster	±
Grey fox	+	Cattle	+
Dog	+	Goats	+
Cat	+	Pigs	+
White rat	±	Wild mice	+
Brown rat	+	Horse	+
Laboratory rabbit	–	Sheep	+
Wild rabbit	+	Baboon	+
Gerbil	±	Other monkeys	+
White mouse	±	Chickens	+
Opossum	+	Goats	+

+ = Treponemes present; ± = treponemes sometimes found; – = treponemes not found.

TABLE II

TREPONEMAL FLORA OF MAN AND ANIMALS (SMIBERT, 1973)[18]

Treponemes	Oral	Genital-anal	Intestinal	Rumen
T. denticola biotype denticola	+	–		
T. denticola biotype commondonii	+	–		
T. oralis	+	–		
T. macrodentium	+	–		
T. vincentii	+	–		
T. scoliodontum	+	–		
T. trimerodontum	+	–		
Rumen fluid requiring strains*	+	–	+	+
T. phadedenis biotype Reiter	–	+		
T. phagedenis biotype Kazan	v	+		
T. refringens biotype refringens	–	+		
T. refringens biotype calligyrum	–	+		
T. hyodysenteriae			+	

+ = Species found; – = species not isolated; blank = no data available; v = occasionally found in simian species.

*Unnamed treponemes requiring rumen fluid from man and animals.

TABLE III

BIOCHEMICAL ACTIVITIES AND DIAMETERS OF TREPONEMES (SMIBERT, 1971., HARIS, 1972., HOLDEMAN, 1972., AND SOCRANSKY, 1969)[15,16,22,23]

Species	Glucose	Lactose	Fructose	Sucrose	Mannitol	Galactose	Cellobiose	Maltose	Mannose	Trehalose	Indol	H_2S	1% Glycine, growth	Lactate used	Esculin hyd.	Diameter of cells, µm
T. phagedenis biotype Reiter	+	+	+	–	+	v	–	–	+	v	+	w	+	–	–	0.25–0.35
T. phagedenis biotype Kazan	+	+	+	–	+	+	–	–	+	v	+	w	+	–	+	0.25–0.35
T. refringens biotype refringens	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+	0.25–0.35
T. refringens biotype calligyrum	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+	0.25–0.35
T. denticola biotype denticola	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+	0.15–0.25
T. denticola biotype commondonii	–	–	–	–	–	–	–	–	–	–	–	+	v	–	+	0.15–0.25
T. oralis	–	–	–	–	–	–	–	–	–	–	+	+		+		0.15–0.25
T. scoliodontum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.10–0.15
T. macrodentium	+	–	+	+	–	v	v	+	–	–	–	+		–	–	0.15–0.25
T. vincentii	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	0.25–0.35
T. hyodysenteriae																0.35–0.45

+ = Positive reaction or weak acid formation, no gas; – = negative test or no acid; v = variable results, some strains +, others –; w = weak reaction.
Note there are no data available for *T. hyodysenteriae*, associated with swine dysentery.