STUDIES ON CELL-MEDIATED IMMUNE RESPONSE TO TREPONEMAL ANTIGENS

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

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

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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.

TABLE IV

END PRODUCTS OF FERMENTATION OF TREPONEMES (SMIBERT,1971., HARIS,1972., HOLDEMAN,1972., AND SOCRANSKY,1969)[15,16,22,23]

Fatty acids	*T. phagedenis*	*T. refringens*	*T. denticola*	*T. oralis*	*T. scoliodontum*	*T. macrodentium*	*T. vincentii*
Acetic	+	+	+	+	+	+	+
Propionic	±	T	±	+	+	–	–
Isobutyric	–	–	–	–	+	–	–
n-Butyric	+	T	T	–	–	–	+
Ethanol	±	–	–	–	–	–	±
n-Butanol	±	–	–	–	–	–	±
n-Propanol	±	–	–	–	–	–	±
Lactic	±	±	+	–	T	+	T
Succinic	T	T	±		T		T

+ = Major end product; ± = minor end product that is usually but not always found; T = trace amounts sometimes found; blank = no data available.

TABLE V

MEDIA USED FOR THE CULTIVATION OF TREPONEMES (BBL., SMIBERT,1971., HOLDEMAN,1972., SOCRANSKY,1969., HARDY,1963.,AND HANSON,1964)[15,22-25]

Ingredients g/L	Spirolate broth	PYGST	E medium	Socransky	Hardy	Hanson
Trypticase (BBL)	15	–	–	–	–	–
Yeast extract	5	10	0.5	–	–	–
Beef extract	–	2	–	–	–	–
Peptone M (Pfizer)	–	20	0.5	–	–	–
Brain-heart infusion broth	–	–	–	–	1 part	45%
PPLO broth without c/v	–	–	–	21	–	–
Spirolate broth	–	–	–	–	3 parts	45%
Tryptone	–	–	–	–	–	0.025%
Glucose	5	10	1.4	1	–	–
Asparagine	–	–	–	–	–	0.025%
Starch	–	–	0.5	–	–	–
NaCl	2.5	–	–	–	–	–
$(NH_4)_2SO_4$	–	0.5	0.5	–	–	–
Gelatin	–	10	–	–	–	–
Agar	–	1.2	1.6	–	–	–
TEM-4T	–	0.16	–	–	–	–
Sodium thioglycollate	0.5	–	–	–	1	0.05%
Cysteine-HCl	1.0	0.9	0.9	1	–	–
Nicotinamide	–	–	–	400 mg	–	–
Spermine tetrahydrochloride	–	–	–	150 mg	–	–
Sodium isobutyrate	–	–	–	20 mg	–	–
Cocarboxylase	–	5 mg	–	5 mg	–	–
$NaHCO_3$	–	5	5	–	–	–
Salt solution	–	500 ml	500 ml	–	–	–
Resazurin solution	–	4 ml	4 ml	–	–	–
Rumen fluid	–	–	280 ml	–	–	–
Rabbit serum	10%	10%	–	–	10%	10%
Water	1000 ml	500 ml	220 ml	1000 ml	–	–
Final pH	7.0	7.2	7.2	7.0	7.2	7.2

Salt solution: K_2HPO_4, 2.25 g; KH_2PO_4, 1.0 g; NaCl, 2 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.2 g; distilled water, 1,000 ml.
Resazurin solution: resazurin, 25 mg; distilled water, 100 ml.
TEM-4T = diacetyl tartaric acid ester of tallow monoglycerides.

TABLE VI

AGAR MEDIA USED FOR ISOLATION AN COLONIAL GROWTH OF TREPONEMES (SCRANSKY,1969.,HANSON,1970., HOLDEMAN,1972., AND SIMBERT,1971)[15,22,23,27]

	Hardy 1	Hardy 2	Hardy 3	H and C(4)	E agar(5)	PYG(6)
USP thioglycollate broth	29.5 g	–	3 parts	–	–	–
Trypticase	–	3%	–	–	–	–
Yeast extract	–	0.5%	–	–	1	10
Glucose	–	0.5%	–	–	2	10
NaCl	–	0.25%	–	–	–	–
Thioglycollate broth	–	–	–	–	–	–
Brain-heart infusion broth	–	–	1 part	45%	–	–
Spirolate broth	–	–	–	45%	–	–
Sodium thioglycollate	–	–	–	0.05%	–	–
Asparagine	–	–	–	0.025%	–	–
Peptone M (Pfizer)	–	–	–	–	1	20
Tryptone	–	–	–	0.025%	–	–
Soluble starch	–	–	–	–	0.5	–
$(NH_4)_2SO_4$	–	–	–	–	0.5	0.5
$NaHCO_3$	–	–	–	–	5	5
Cysteine-HCl	–	0.2%	–	–	0.8	0.8
Calf serum	10%	–	–	–	–	–
Rabbit serum	–	10%	10%	10%	–	10%
Bacto-agar	–	–	–	2.5%	–	–
Ionagar No. 2	0.7%	0.7%	0.7	–	0.7	0.7
Salt solution	–	–	–	–	500 ml	500 ml
Rumen fluid	–	–	–	–	280 ml	–
Distilled water	1000 ml	1000 ml	1000 ml	–	280 ml	500 ml
pH	7.2	7.2	7.2	7.2	7.2	7.2

Salt solution: K_2HPO_4, 2.25 g; KH_2PO_4, 1 g; NaCl, 2 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.2 g; distilled water, 1,000 ml.

1 = Medium for Reiter, Kazan A, and 5 oral treponemes,
2 = Medium for Noguchi, Nichols, and other Kazan strains,
3 = Medium for FM oral treponeme and N-9 strain of *T. vincentii*.
4 H and C = Hanson and Cannefax,
5 and 6 = E Agar and PYG agar used in the author's laboratory. In g/L

ULTRASTRUCTURAL STUDIES OF TREPONEMES

The treponemes have a basic morphology consisting of an outer envelope (OE)*, axial filaments, and a cell wall-membrane complex enclosing the protoplasmic cylinder.

It is very difficult to differentiate the virulent Treponema pallidum (Nichols strain) from those non-virulent treponemes by morphological studies. A degree of uncertainty exists as to whether virulent Treponema pallidum is morphologically similar to the non-pathogenic treponemes and other spirochetes. The uncertainty is associated with the presence or absence of an outer envelope in the virulent strain.

Ovcinnikov and Delektorskij (1971)[28] employed the technique of electron microscopy for the morphological study of T. pallidum and found that the outer wall of the organism consisted of three layers. The axial filaments lie between the outer envelope and the cytoplasmic membrane which also has three layers. In contrast to these findings, Drusin et al., (1969)[29], Azar et al., (.970)[30], and Hasegawa (1969)[31] found that the axial filaments of T. pallidum were external to the body of the organism and failed to reveal any outer trilaminar structure using electron microscropy. More recently, Wiegand et al., (1972)[32] also failed to find trilaminar membrane external to the axial filaments in T. pallidum from a human penile chancre and pathogenic T. pallidum(Nichols strain) from rabbit testes. Sykes and Miller (1972)[33] supported this finding by comparing the ultrastructural morphology of virulent T. pallidum (Nichols)

* Various authors use envelope (ENV), outer envelope (OE), outer structure or outer wall to describe the structure outside of peptidoglycan of treponemes. Therefore envelope (ENV), outer structure and outer wall are synonymous and interchangeable in this dissertation.

with two representative non-pathogenic treponemes, namely, T. reiter and T. denticola, prepared by ultracentrifugation for electron microscopic studies. Table VII[33] showed the different dimensions of T. pallidum (Nichols), T. denticola and T. reiter. The outside diameter of T. denticola was estimated to be 224.9±2.89 nm, and that of T. reiter was 331±4.15 nm, contrasting with T. pallidum (Nichols strain) which has a marked smaller diameter of 163.0±1.9 nm for lack of trilaminar membrane external to the axial filaments.

All these findings suggest that virulent T. pallidum might be morphologically different from some of the non-pathogenic treponemes in having the axial filaments as the outer most structure devoid of a surrounding trilaminar membrane, as demonstrated in the structures of T. reiter and T. denticola. However, Johnson et al., (1973)[34] was able to demonstrate trilaminar outer structure of T. pallidum by using direct rabbit testicular tissue for ultrathin electron microscopic studies in contrast to Skyes and Millers' technique of collecting the organisms by ultracentrifugation following extraction of minced tissue.

Johnson's direct observation seems to be less destructive to the treponemal organisms and more reliable.

The following evidence indicates trilaminar outer structure of treponemes to be lipopolysaccharide (LPS) in nature:

1) Thin section microscopic studies[35] show that the treponemes and gram-negative bacteria are very similar in ultrastructure (figure 1)[35]. Both types of cells are surrounded by an inner unit cytoplasmic membrane (PM)[35]. The intermediate layer of treponemal surface, situated just external to the cytoplasmic membrane, has been shown to contain the

TABLE VII

COMPARISON OF SOME DIMENSIONS OF TREPONEMA PALLIDUM (NICHOLS), T.DENTICOLA AND T. REITER (SYKES AND MILLER, 1973)[33]

Diameters (D) and width (W)[b]	Dimensions of treponemes (nm)[a]		
	T. denticola	*T. reiteri*	*T. pallidum*
D1	224.9 ± 2.83	331.0 ± 4.15	—[c]
D2	203.1 ± 2.9	309.0 ± 4.7	—
D3	179.5 ± 3.0	263.4 ± 8.5	163.0 ± 1.9
D4	162.5 ± 3.4	261.0 ± 4.43	137.0 ± 3.2
D5	19.2 ± 0.2	18.0 ± 0.35	31.0 ± 0.73
W1	5.5 ± 0.16	7.17 ± 0.39	—
W2	5.4 ± 0.31	3.9 ± 0.55	—
W3	5.0 ± 0.16	3.4 ± 0.50	—
W4	8.3 ± 0.38	11.8 ± 0.53	—
W5	4.6 ± 0.28	6.5 ± 0.23	6.5 ± 0.15
W6	6.7 ± 0.26	8.5 ± 0.29	6.7 ± 0.18

[a] Measurements in nanometers (nm) are followed by the standard deviation. Measurements were taken from electron micrographs of the organisms photographically enlarged to × 140,000. Only "right" cross sections were measured, and the standard deviation was calculated from a minimum of 50 and a maximum of 100 measurements.

[b] See diagram 1.

[c] Dash (—) indicates that these structures were not seen in electron micrographs of right cross sections of *T. pallidum* (Nichols strain).

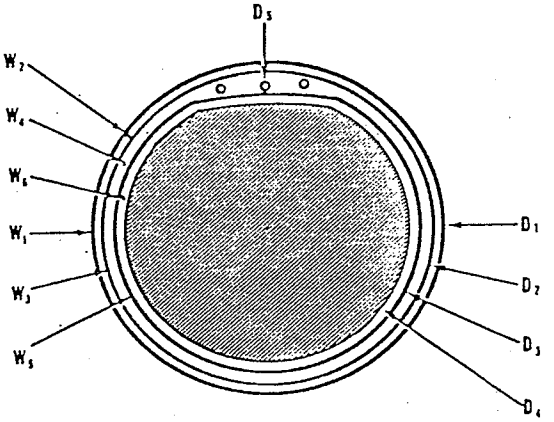
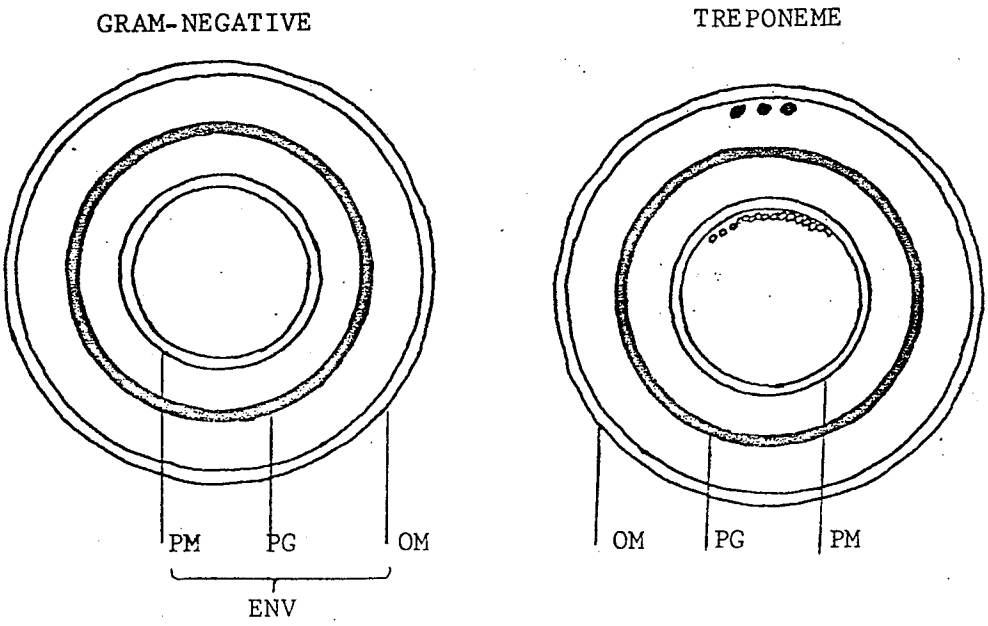


Diagram: Right cross section through a treponeme. D_1–D_5 indicate diameters and W_1–W_6 indicate the width of the structures indicated by the arrows. Arrows terminating in an "O" refer to electronlucent spaces.

Figure 1

Diagrammatic representation of a cross-section of both a gram-negative organism and a treponemal cell. Abbreviations: PG, peptidoglycan; PM, plasma membrane; OM, outer membrane; ENV, envelope. (Osborn, 1971)[35]



peptidoglycan (PG) which are similar to gram-negative bacteria.[36] The outer unit membrane (OM) external to peptidoglycan of gram-negative bacteria is well established to be the LPS-containing layer. While the outer envelope of treponemes were shown to have the characteristics of outer membrane of gram-negative bacteria in their ultrastructures.
2) Pillot (1965)[36] isolated lipid, protein, and carbohydrate from the envelope of the Reiter strain of treponema. An envelope consists of these components is compatible with an LPS-containing structure.
Shands (1971)[37] extracted LPS from Nichols, non-pathogenic T. pallidum, by phenol-water procedure and demonstrated a variety of shapes of LPS under electron microscope. Such a polydispersity of shapes are characteristic of bacterial lipopolysaccarides. Jackson and Zey (1972)[38] reported that the chemical similarity between the LPS from Nichols, non-pathogenic strain of T. pallidum, and from the gram-negative bacteria is indicated by the positive reaction of treponemal LPS in the colorimetric assay for lipopolysaccharides.

The importance of the LPS has been demonstrated by Pillot (1965)[36] by isolating polysaccharides from the envelope of Reiter strain and showed that it was responsible for both immunogenicity and antigenic specificity of that strain. Moreover, he suggested that this specificity of the envelope is analogous to the specificity by which species of Salmonella are serotyped.

CHEMICAL COMPOSITIONS OF TREPONEMES

Johnson et al. (1970) reported that in Kazan-5, 18 to 20% of the dry weight was lipids, which is higher than those in most other bacterial cells.[39] It was also found that lipid and fatty acid are the most important compositions in treponemes.[39, 40] Monogalactosyldiglyceride was found in all treponemes and C-16:0, C-18:1, C-18:2 and C-18:0 were the main fatty acid[16], (Table VIII.[39, 41, 42] Meyers (1970) demonstrated that Reiter strain can neither synthesize nor desaturate fatty acids and depends on outside sources for its fatty acids. In contrast, the free-living Treponema zuelzerae can sythesize all of its fatty acids, primarily iso-acids and straight-chain saturated fatty acids plus smaller amounts of monoenoic acids and free fatty aldehydes. Reiter strain and T. zuelzerae can synthesize all of their complex lipids. Glycolipids account for one-fourth to one-third of the total lipids in both organisms, principally monogalactosyl diglyceride and monoglucosyl diglyceride. The major phospholipids of T. zuelzerae are phosphatidyl glycerol (31%) and cardiolipin (21%) while Reiter strain contains phosphatidyl choline (33%), choline plasmalogen (8%), phosphatidyl glycerol (7%) and cardiolipin (6%).[41] Johnson et al., (1970)[39] studied the lipids of Treponema pallidum Kazan 5 and showed that in Kazan 5, glycolipid and phospholipids accounted for 90 to 95% of the total lipids and free fatty acids made up the remaining 5 to 10%. The major polarlipids were the glycolipid, 1-(O-α-D-galactopyranosyl)- 2,3-diglyceride (45-55%), and phosphatidylcholine (30 to 40%), phosphatidylethanolamin (5 to 10%), an uni-

dentified compound (1 to 2%), and occassional trace amounts of diphosphatidyl glycerol (cardiolipin) were also found.

Using gas-liquid chromatography (GLC), Moss (1971) studied the amino composition of whole cell hydrolyates of fourteen different cultivable treponemes to show that generally the main amino acids in the treponemes are alanine, leucine and aspartic acid.[43] However, the relative molar ratio of amino acids revealed no qualitative or sufficient quantitative difference among the strains.

Nell and Hardy (1966) isolated polysaccharides from the Reiter strain by fractionation of an alkaline digest of treponemes ghost. The chemical analysis showed that the polysaccharide was composed of simple sugars, amino sugars and a small amount of amino acid residues. Amino sugar, expressed as glucosamine amounted to 27% of the antigen. Exclusive of amino sugars, total carbohydrate calculated as glucose, accounted for 42% of the total weight by the indole reaction and 64% by the orcinol-H_2SO_4 reaction. Moreover, ketose (calculated as fructose) was found to be 18.5 per cent. The individual sugars identified chromatographically were glucose (16.5 per cent), galactose (25 per cent) and rhamnose[44]. Heptoses, 2-deoxypentoses, uronic acids or sialic acid were not present.[45]

Very little is known about the fermentation of amino acids and related enzyme systems of various species of treponemes as compared to the understanding of fermentation of sugars.

Hespell (1971) found that T. denticola fermented glucose by the Embden-Meyerhof pathway to produce a small amount of fermentation end products containing acetate, lactate, succinate, formate, pyruvate,

ethanol, CO_2, H_2O, and NH_3. Amino acids were fermented and served as the major energy sources for the organism.[46] Some enzyme systems of treponemes are listed in Table IX.[18] Barban (1954)[47] reported the following findings in his transamination study of Reiter strain: a) the presence of a diphospho-pyridine nucleotide dependent glutmatic dehydrogenase; b) L-glutamate, L-histidine, L-cysteine, L-arginine, and L-threonine were shown to be deaminated in Reiter strain; c) carbon dioxide was produced only from L-glutamate and L-histidine; d) glutamate was metabolized anaerobically giving rise to ammonia, carbon dioxide, and succinate as the chief end products. Reiter and Kazan were reported by Tauber (1962)[48] to contain much cysteine desulfhydrase in addition to moderate quantities of the other enzymes, including a DPN-dependent isocitric-dehydrogenase but not TPN-dependent isocitric dehydrogenase. However, the Treponema pallidum did not have acetokinase, aceto-CO-A-kinase, phosphotransacetylase, or β-galactosidase activity.

The Reiter treponema is the most studied strain and it requires glucose as an energy source. Glucose is utilized to consist 28 to 43% of cellular material in this particular strain. Arginine, histidine, serine, threonine, and glutamate are degraded and supply 41 to 54% of cellular material. Moreover, fatty acids in media were responsible for 18% of cellular material. Reiter treponema consist of 1.8% DNA and 10.2% of ribonucleic acid (RNA); the percentage of guanine and cytosine in DNA is 38%. Adenine, guanine, cytosine, and uracil are incorporated into cells and are not fermented. Thymine in the medium is not utilized by Reiter treponema.[49]

TABLE VIII

LIPIDS FOUND IN TREPONEMES AND SPIROCHAETA (CONLON-MORELEC, 1969., MEYER, 1970., AND JOHSON, 1970)[26,41,42]

Lipids Found in Treponemes and Spirochaeta

	Reiter	Reiter	Reiter	Kazan-5	Noguchi	Nichols*	*S. zuelzerae*
Phosphatidylcholine	+	+		+			–
Lyso-phosphatidylcholine	±	–		–			–
Phosphatidylglycerol	±	+		–			+
Cardiolipin	±	+		±			+
Lyso-cardiolipin	±	–		–			±
Unidentified phospholipid	±	±		–			±
Mono-galactosyldiglyceride	+	+	+	+	+	+	–
Lyso-galactosyldiglyceride	±	–		–			–
Mono-glucosyldiglyceride	–	–		–			+
Lyso-glucosyldiglyceride	–	–		–			+
Unidentified glycolipid	±	±		±			±
Phosphatidylethanolamine	–	–		+			–

+ = Lipid present; ± = small amount found; – = not found; blank = not reported.
Data on Reiter strain from 3 different reports.
Note: neutral lipids are also present as well as free fatty acids and aldehydes.
*Avirulent cultivated Nichols strains.

TABLE IX

ENZYMES FOUND IN TREPONEMES (SMIBERT, 1973)[18]

	T. denticola	Reiter	Kazan-2	Kazan-4	Kazan-5	*T. pallidum*
Hexokinase	+					
Glucosephosphate isomerase	+					
Phosphofructokinase	+					
Fructosediphosphate aldolase	+					
Glyceraldehydrophosphate dehydrogenase	+					
Triosephosphate isomerase	+					
$NADH_2$ oxidase	+	+				
Catalase	–	–	–	–	–	
Oxidase	–	–	–	–	–	
Succinic dehydrogenase		+				
Phosphotransacetylase	+					
Acetokinase	+	+	+	+	+	–
β-Galactosidase		+	+	+	+	–
Aceto-CoA-kinase		–	–	–	–	–
Threonine aldolase		+				
Lactic dehydrogenase				+		
Ethanol dehydrogenase				+		
Isocitric dehydrogenase		+	+			
Glutamate dehydrogenase		+				
Phosphotransacetylase		+	+	+	+	–
Cysteine disulfohydrase		+	+			
Transamination of α-ketoglutarate by alanine		+				
arginine		+				
aspartate		+				
histidine		+				
threonine		+				
Transamination of glyoxylate by glutamate		+				

+ = Activity found; ± = very weak activity reported; – = no activity.

ANTIGENIC COMPONENTS OF TREPONEMES

In treponemes, the antigenic activity is associated with lipid, proteins and polysaccharides, In order to study the antigenic relationships among the treponemes, antiserum of elicited by every strain was tested immunologically with all strains to study the degree of cross reaction.

The antigenic components of treponemes are discussed as follows:

1) The cardiolipid hapten

In 1906, Wassermann utilized the aqueous extract of the liver of the congentially syphilitic newborn to fix complement with the syphilitic sera while the same liver extract failed to fix the complement of the normal serum.[50] Marine and Levaditi reported the same fixation results using normal liver extract.[49] The nature of this immunological reaction was established during the period of 1940 to 1950. The aqueous extract of liver, normal or syphilitic, is a lipidic fraction which contains a hapten that reacts with the antibody called reagin in syphilitic sera.

Cardiolipid hapten is widespread. It can be extracted from all tissues of mammals including heart and liver. It has also been found in the vegetable kingdom (cabbage, carrots, peas, and wheat sprouts), in bacteria, as well as in Treponema reiter.[51] The immunogenicity of Wassermann hapten was weak unless it is coupled with protein, while reagin antibody can be induced by all treponematosis.

Pangborn (1964) found the purified hapten to be a phosphatide. The hapten is called cardiolipin to indicate the main source of isolation. The exact location of cardiolipin in the treponema is still unknown. The cardiolipin from Treponema pallidum was demonstrated in the agglutination reaction.[52] However, it is difficult to free the cardiolipin of T. pallidum

from the contamination of rabbit tissuses.

2) The protein antigens

Pillot and Faure (1959) suggested that ultrasonication was the best way to obtain the soluble protein antigen by breaking the cell wall of treponemes. It was superior to those of cryolysis or grinding with glass beads. Although contamination of the cytoplasmic soluble protein from cell wall substance is inevitable, the soluble protein antigen is valid for immunological analysis such as gel diffusion.[53]

The yield of this soluble antigenic protein varied in different strains. For example, large amount of this antigen was isolated from T. phagedenis, and smaller amount was found in T. refringens, T. calligyrum, and T. minutum.[54]

To demonstrate that the soluble protein antigen is a common antigen among treponemes, Treponema reiter was used to absorb the antisera which induced by cultivated oral and genital treponemes (Treponema denticola, T. vincentii, Reiter, etc.), and the reactivity to T. pallidum was removed indicating the removal of the common antigen by Reiter strain.[55] At the present time the antigen appears to be shared by all the strains of the Treponema genus.

3) The polysaccharide antigens

The envelope of T. reiter is rich in polysaccharide which may or may not be linked to lipids. By means of diffusion precipitation test, it has been indication that the envelope of T. reiter contains approximately two third of the antigens. In complement fixation test, it has also been shown that all the immunological specificities of the envelope is linked to the polysaccharides. It was further observed that only after the alteration or dispersal of the envelope, the cross-reactions between antisera prepared

by different species of treponemes appeared.[56]

Using diffusion precipitation test, Pillot and Dupouey (1964) analyzed the different polysaccharide extracts from treponemes. The results have shown that 5 to 6 fractions, at minimum, can be demonstrated in T. reiter. However, the contamination of protein is in large amount.[57]

Christiansen (1964) observed that the Reiter and Kazan treponemes were immunochemically very similar but not identical. The Kazan 2 strain has been shown to have two phenol-water extractable polysaccharide antigen which is not possessed by the Reiter strain.[58] Miller et al., (1966, 1969) purified an ultracentrifugally homogeneous heat-stable polysaccharide from the virulent Nichols strain. Free of rabbit tissue and cardiolipin, this heat-stable polysaccharide was found to react with Nichols strain antisera but not with human syphilitic antisera. The polysaccharide was strain specific and was not shared by Reiter treponema.[59, 60] Nell and Hardy (1966) extracted a purified polysaccharide from Reiter treponema. The purified polysaccharide antigen reacted with antisera to the Kazan strains. Two oral treponemes and one strain of Borrelia were in an identical fashion as compared to the reaction with the homologous antisera, while antisera to the Noguchi, non-pathogenic Nichols strain were non-reactive with the Reiter polysaccharide.[61]

Once pure treponemal polysaccharide are isolated, a detailed study for the classification and differentiation of the various strains can be carried out.

By means of agglutination and complement fixation test, the following results are listed in Table X:[62,63]

1) The Treponema phagedenis, the Reiter and all Kazan strains are closely related, but not identical.

2) The Nichols and Noguchi strains are serologically indistinguishable.

3) The T. refringens, T. calligyran, Nichols and Noguch strains are cross-reacted.

4) T. denticola strains are cross-reacted among themselves.

5) The Kroo strain is serologically distinct from other treponemes.

6) T. minutum only weakly cross-reacted with some treponemal strains.

In the immunofluorescence studies for the antigenic grouping of cultivable treponemes, antisera were prepared against each treponemal strains, and the immunoglobulins were conjugated with fluorescein isothiocyanate. The results are summarized in Table XI:[61]

1) All reactivities were removed in the Reiter and English Reiter system after the absorption of the immunoglobulins-fluorescein isothiocyanate conjugated with Reiter strain. The absorbed conjugate prepared for the five Kazan continued to react with Kazan antigens with a greatly decreased of reactivity.

2) Absorption of the anti-Reiter conjugate with Kazan strain reduced, but did not eliminate reactivity with Reiter and English Reiter. All reactivities were removed from the five Kazan strains conjugates after cross absorption with Kazan strain. All these findings indicated that Reiter and Kazan strains are cross reacted but not identical.

3) Absorption of T. denticola N-39 conjugated with T. denticola FM remained the reactivities for N-39.

4) Absorption of T. denticola MLB conjugated with N-39 rendered the reactivities for FM and MRB.

It is suggested that treponemes possess their own specific antigens but the location of these antigens are not well defined. Further investigations are needed to determine different parts of treponemes and its chemical and antigenic composition.

TABLE X

IMMUNOLOGICAL RELATIONSHIPS OF TREPONEMES (DUPONEY, 1963., AND EAGLE, 1948)[62,63]

Organism	Serogroup: *T. phagedenis* Reiter Kazan Kazan-2 to 8	*T. refringens* Nichols Noguchi *T. calligyrum*	Kroó	*T. denticola* MRB FM N-9	*T. vincentii* N-9	*T. minutum*
T. phagedenis	+	–		–	–	∓
Reiter	+	–	–	–	–	∓
English Reiter	+	–		–	–	∓
Kazan	+	–	–	–	–	
Kazan 2	+	–		–	–	
Kazan 4	+	–		–	–	
Kazan 5	+	–		–	–	
Kazan 8	+	–		–	–	∓
T. refringens	–	+		–	–	
Nichols	–	+	–	–	–	
Noguchi	–	+	–	–	–	∓
T. calligyrum	–	+		–	–	
T. minutum	–	–		–	–	+
Kroó	–	–	+	–	–	
T. denticola MRB	–	–		+	–	
T. denticola FM	–	–		+	–	
T. denticola N-39	–	–		+	–	
T. vincentii N-9	–	–		–	+	

+ = Cross reacts; ∓ = weakly cross reacts; – = no cross reaction; blank = no data available.

Note: Most strains will agglutinate each other's sera due to possession of a common antigen on cells. Most data in table derived sera absorbed with Reiter treponeme to remove common antigen.

TABLE XI

SEROTYPING OF TREPONEMES (NELL, 1966)[61]

Organism	Sera adsorbed with Reiter antigen			Sera adsorbed with Kazan		Sera adsorbed with FM	Sera adsorbed with N-39
	Reiter	E. Reiter	Kazan	Reiter	Kazan	N-39	MRB
Reiter	–	–	–	+	–	–	–
English Reiter	–	–	–	+	–	–	–
Kazan	–	–	+	–	–	–	–
Kazan-2	–	–	+	–	–	–	–
Kazan-4	–	–	+	–	–	–	–
Kazan-5	–	–	+	–	–	–	–
Kazan-8	–	–	+	–	–	–	–
T. denticola N-39	–	–	–	–	–	+	–
T. denticola MRB	–	–	–	–	–	–	+
T. denticola FM	–	–	–	–	–	–	+

+ = Positive serologic reaction; – = no serologic reaction.

MATERIALS AND METHODS

MICROORGANISMS

The cultivable Treponema phagedenis biotype English Reiter; Treponema phagedenis biotype Kazan 4; and Treponema phagedenis biotype Kazan 5 were obtained from National Center for Disease Control of Ottawa while Treponema refringens biotype refringens; Treponema refringens biotype Nichols; Treponema denticola; and Treponema scoliodontum were obtained from Dr. R. C. Johnson, University of Minnesota. The non-cultivable pathogenic Treponema pallidum was obtained from Dr. J. N. Miller, University of Los Angeles.

CULTURE MEDIUM FOR CULTIVABLE TREPONEMES

The modified Hanson and Cannefax liquid medium[25] was used for the cultivation of treponemes. The composition of the liquid medium is given below:

45%	NIH Thioglycolate broth	v/v (BBL)
45%	Brain Heart Infusion	v/v (BBL)
0.05%	Sodium thioglycolate	w/v (Difco)
10%	inactivated (56°C for 30 min.) normal rabbit serum	v/v

The medium was dispensed into 500 ml bottle and autoclaved at 121°C for 15 min., cool to room temperature and add the sterile inactivated normal rabbit serum. The final pH was adjusted to 7.2. The medium was stored in tightly closed containers at room temperature and was used within five days after preparation. Sterility tests of the culture medium were made on blood agar plates and in a beef broth medium.

CULTURING OF TREPONEMES

The treponemes were maintained by subculturing in the liquid medium.

A heavy growth culture was used as the inoculum. The purity of the cultures was checked by examining smears strains by Gram's method and Fontana's method at 1000× magnification microscope and by cultural tests. Cultural tests were performed on blood agar plates and in the beef broth medium.

The treponemes were grown in Hanson and Cannefax's modified liquid medium at 37°C until visible heavy growth were observed, usually seven days. Each bottle, containing 400 ml of the medium, was inoculated with 20 ml of suspension of cells from the heavy growth culture. The purity of the cultures was checked as above.

PREPARATION OF CYTOPLASMIC ANTIGENS OF CULTIVABLE TREPONEMES

Pure cultures of treponemes were treated with few drops of 1% sodium azide. They were left at room temperature for overnight, and then centrifuged at 23,500 g for 15 min. in an International Refrigerated Centrifuge Model B-20 (International Equipment Co., Mass.,U.S.A). The sediments were washed few times with 0.85% phosphate buffered saline (PBS) until no trace of protein was revealed in the washings as measured at 280 nm wavelength in the Unicam Sp 1800 Ultraviolet Spectrophotometer. To the final washing, five times volume of 10^{-5}M EDTA (ethylene diaminetetraacetic acid) containing 100 units penicillin per ml, 50 µg streptomycin per ml,pH 7.4 was added and the suspension was sonicated by a Bronwill Biosonik III Ultrasonicator (Bronwill Scientific, Rochester, N.Y., U.S.A.) for 20 minutes at 40 kc/s until most of the cells were disrupted, as shown by dark field microscopy. After the disintegration, the cell debris and cytoplasmic membranes were removed by centrifugation at 23,500 g for 20 min. The supernatant was collected and recentrifugated at the same

speed for 10 minutes one or more times until no particular material was found microscopically in smears prepared from the supernatant. This material represented the cytoplasm preparation and was lyophilized before used for the immunization, intradermal challenge, and macrophage migration inhibition assay.

PREPARATION OF CYTOPLASMS OF TREPONEMA PALLIDUM

Rabbits were injected intratesticularly with 0.5 ml of a PBS (phosphate buffered saline) suspension of 10^8 live cells of Treponema pallidum in each testicle.

When orchitis developed (between 18-22 days) the rabbits were bled and the testicles were removed. The testicles proper were then excised from the overlaying tissues, placed in a triple volume of PBS (pH 7.4) with 1.3M sucrose medium, homogenized in a Sorvall Omni-mixer at the lowest speed for 30 seconds, and centrifuged at 900 g in order to remove cell debris. At each step,aliquot of the material was examined at 600 x magnification by the dark field microscope. The supernatant was centrifuged for one hour at 17,000 g, in order to separate treponemes from fine cell debris. The supernatant, containing treponemes was then collected and dialysed overnight against PBS (pH 7.4). The dialysate was concentrated to 1/10 of the original volume of the tissue suspension. The partly purified treponemes were then disrupted by ultrasonicating them at 20 kc/s for 3 min. in a cold ice bath. The material thus obtained was centrifuged at 760 g for 10 min., and the supernatant was gently withdrawn and recentrifuged as above. The final fluid representing the partly purified cytoplasm of Treponema pallidum was used for intradermal challenge on sensitizing

rabbits. The cytoplasm was filtered through a 0.45 u membrane filter before it was used.

PREPARATION OF EXTRACTS FROM NORMAL RABBIT

A normal non-infected rabbit was bled out; the testicles were removed and the fatty tissue was removed from above the testicles proper. The defatted testicles were then cut into 2-3 mm thick slices and suspended in a triple volume of sterile distilled water. After homogenized in a Sorvall Omin-mixer at the lowest speed for 3 min. it was centrifuged at 15,000 g for 20 min. The supernatant fluid which represented the normal rabbit testicle extract, was filtered through a 0.45 μ membrane and was used for the skin tests.

DERMAL HYPERSENSITIVITY ASSAY

The dermal hypersensitivity assays were carried out in guinea pigs and rabbits. For these studies, an Albino strain of randomly bred guinea pigs, weighing from 600 to 800 grams, were used for both the Macrophage Migration Inhibition (MMI) test and for skin test. By means of a VDRL test (Venereal Disease Research Laboratory), they were found to be non-reactive with cardiolipin. Rabbits used were New Zealand Albino males, weighing from 1.5 to 3.0 kg. The rabbits were first screened for the presence of reagins by means of a VDRL test and inspected carefully for any skin lesions. They were caged individually in a seperate room, at the temperature of 18^{o}C.

Guinea pigs were sensitized with the treponemal cytoplasms in the following procedure: one ml of aliquot containing 300 μg of lyophilized cytoplasm of cultivable treponemes, 100 units penicillin and 50 μg strepto-

mycin with a pH 7.4 was injected into each guinea pig. Twelve guinea pigs were sensitized with a single batch of cytoplasm and a total of 84 guinea pigs were used for seven different cytoplasms, i.e., T. 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. For the sensitization of guinea pigs, 0.1 ml of aliquot containing 30 μg of lyophilized cytoplasm was injected into each of the hind footpads and 0.8 ml of aliquot containing 240 μg of cytoplasm were injected subcutaneously into the back of the guinea pigs.

Rabbits were injected intratesticularly with 0.5 ml of a PBS suspension containing 10^8 live cells of Treponema pallidum, in each testicle. When orchitis developed (between 18-22 days), the rabbits were used for dermal hypersensitivity tests.

SKIN TEST ON GUINEA PIGS

The suspensions of 100 μg/ml of lyophilized cytoplasm of cultivable treponemes in 10^{-5} M EDTA containing pencillin and streptomycin at pH 7.4, were used for the intradermal challenge. Prior to the dermal hypersensitivity assays, possible toxicity of the challenging antigens was examined in the skin of normal guinea pigs and were found not to be injurious to the skin.

For the test proper, four guinea pigs were used for each cytoplasm, on the 12th, 20th, and 30th day after the sensitization, the skin of guinea pigs was prepared by shaving their back and applying the hair remover "Neet" (Whitehall Laboratories Limited, Toronto, Canada) to remove fine hair.

As a challenge, 0.2 ml of the challenging antigens were injected in two different sites (0.1 ml/site) or each guinea pig. As a control, 0.1 ml of sterile saline was injected into one site. The skin test sites were examined at 24, 48, and 72 hours. The sites were inspected for the presence of erythema and induration; and dimensions of the indurated areas were measured in two directions. Average areas of induration were computed for the test sites corresponding to each antigen. Since induration was irregular in shape the measurements were standardized by multiplying the longest axis by the shortest axis. The product was referred to as the area of induration. Areas of induration smaller than 40 mm^2 were considered insignificant. Skin plugs were removed at various times after the challenge, and Fixed in Bouin's fixer. The tissues were then processed by routine histological methods.

SKIN TEST IN RABBITS

Two infected rabbits started to show orchitis were used for skin testing. The skin test antigens were the cytoplasms of cultivalbe treponemes (T. 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), The cytoplasm of the partly purified Treponema pallidum, and the normal rabbit testicle extraction. The skin tests of rabbits were carried out as the same as in the guinea pigs.

MACROPHAGE MIGRATION INHIBITION (MMI) TEST

The suspensions of 100 μg/ml of lyophilized cytoplasm from cultivable treponemes in 10^{-5}M EDTA (with penicillin and streptomycin) at pH 7.4, were used for the macrophage migration inhibition tests. All

the antigens had reacted with macrophages from normal guinea pigs and were non-toxic to the normal macrophages.

Guinea pigs were injected intraperitoneally with 30 ml sterile 2.5% of soluble starch gel (Difco, Michigan, U.S.A.) three days before the peritoneal cells were harvested.[64] The animals were skin-tested before their macrophages were used in the MMI tests. The guinea pigs were sacrificed by bleeding out by cardiac puncture. Chloroform was not used as it tends to have a detrimental effect on the metabolism of the macrophages.[65] The peritoneal cavity of guinea pigs was washed with 100-150 ml of sterile Hank's balanced salt solution. The abdomen was massaged and then opened. A sterile pasteur pipette was inserted into the peritoneal cavity, and gentle suction was used to collect the exudate into a 100 ml silicone treated glass centrifuge tube. The exudate was then centrifuged at $4^{o}C$ for 10 minutes in a IEC International Centrifuge (Universal Model UV) at 1200 rpm. The cells were washed twice with sterile Hank's BSS and centrifuged for 5 min. at 100 rpm at $4^{o}C$. To 0.1 ml packed cells, 0.5 ml 199 tissue culture medium containing 15% normal guinea pig serum was added. The cell suspension was kept in an ice-bath to minimize the cellular metabolism. Capillary tubes (50 µl) were filled with the cell suspension and sealed at one end by non-drying modeling clay (Peter-Austin MFG. Co., Toronto, Canada). The tubes were spun at 900 rpm at room temperature for 5 minutes in an IEC centrifuge. The capillary tubes were then cut slightly below the packed cell-liquid interface and fastened inside an incubating chamber (1.5 cm in diameter and 0.2 cm deep) with silicone grease. One capillary was placed in each chamber. The

chambers were then filled with approximately 0.6 ml of 199 tissue culture medium containing 15% inactivated normal guinea pig serum and 0.1 ml of cytoplasm antigen. The chamber was then covered with a sterile cover glass and sealed with grease. The chambers were incubated at 37°C for 24 hours. The area of migration was mapped by projection microscopy (Nikon Profile Projector, Model 6C, Nippon Kogaku K.K., Japan) and measured by planimetry (K+E, Model 62002, Keuffel and Esser Co., Germany). The macrophage inhibition (MMI) indices and the area of migration was expressed as below 9, 2:

$$MMI = \left(1 - \frac{\text{area of migration with antigen}}{\text{area of migration without antigen}} \right) \times 100$$

For each individual test, the final MMI index was calculated by the average of four capillaries.

STATISTICAL ANALYSIS

The program of mixed factoral design[66] was applied for the study of skin test and macrophage migration inhibition test. Two independent factors were involved in both tests while two correlated factors were concerned in skin test but only one correlated factor for macrophage migration inhibition test. The two independent factors consisted of seven sensitizing strains (T. 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) and three different days for skin challenge (12, 20, and 30 days). The correlated factors were two related times for the measurements of skin reactions (24 and 48 hours after intradermal challenge) and one related time for

the examinations of macrophage migration inhibition (24 hours after incubation) and eight related antigens for skin test and macrophage migration inhibition test. These antigens were T. scoliodontum, T. phagedenis biotype English Reiter, T. phagedenis biotype Kazan 5, T. phagedenis biotype Kazan 4, T. denticola, T. refingens biotype Nichols, T. refringens biotype refingens, and saline as the control.

All tests were repeated four times. The analysis of variance was used.[66] As unequal numbers of subjects were presented in each group, the means were weighted. The level of significance was = 0.05.

The mutual relationships among treponemal antigens in skin test and macrophage migration inhibition test were determined by the Duncan's New Multiple-Range test[67] (X = 0.05) and by a simple comparative percentage calculation using antigens as one hundred percent by turns.

RESULTS

I. Dermal hypersensitivity assay

The computer analysis of variance on the data obtained from the dermal hypersensitivity testing are summarized in Table XII, from which the following observations are made:

Dermal reactions of guinea pigs after immunization with different strains.

Extent of skin sensitization showed statistically significant (α = 0.05) differences. Different strains gave rise to different degrees of sensitization (F=7.595).

The sensitized animals showed difference in the extent of skin reaction which varied significantly (α = 0.05) depending on the period of sensitization (namely, 12, 20, and 30 days) and on the strain used. Relations between individual strains and the period of sensitization are different (F = 7.814).

Heterologous antigens used for the intradermal challenge on the sensitized animals produced significantly (α = 0.05) different skin reactions (F = 88.884).

Animals immunized with different strains gave rise to significantly (α = 0.05) different response to different skin test antigens. The relations between each strain and skin test antigens were different (F = 11.779).

Comparison of the degree of sensitization among strains.

The data in tables are expressed in cm^2 but they have been changed into mm^2 in the test, for an easier discussion.

Table XII presented the results of means and standard errors of skin reactions. The means of skin reaction of each strain was computed from 192 readings (four replications from two related times, 24h and 48h,

TABLE XII

ANALYSIS OF VARIANCE OF THE NUMERICAL DATA
OBTAINED FROM DERMAL HYPERSENSITIVITY ASSAYS

SOURCE OF VARIATION	DF	SS	MS	F
STR	6	4.8217	0.8036	7.595
STR DAY	12	9.9211	0.8268	7.814
CHA	7	14.1895	2.0271	88.884
STR CHA	42	11.2823	0.2686	11.779

STR: strains for sensitization

CHA: challenging antigens

DF: degree of freedom

SS: sum of square

MS: mean of square

F: F ratio

TABLE XIII

MEANS AND STANDARD ERRORS OF THE DATA FROM HYPERSENSITIVITIES TO ALL CYTOPLASMS IN GUINEA PIGS SENSITIZED WITH THE CYTOPLASM OF INDIVIDUAL STRAINS

	N	MEAN	S.E.SQRD	S.E.
STR				
1	192	0.257	0.001	0.023
2	192	0.340	0.001	0.023
3	192	0.271	0.001	0.023
4	192	0.279	0.001	0.023
5	192	0.336	0.001	0.023
6	192	0.440	0.001	0.023
7	192	0.271	0.001	0.023

STR: cytoplasm of all sensitizing strains
1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens

N: number of readings

S.E.SQRD: standard error of square

S.E: standard error

in three different days, 12, 20, and 30 respectively (4 x 2 x 3), seven strains plus control (8). It could be seen that T. refringens biotype Nichols induced the strongest skin reactions in animals by having an average 44 mm^2 area of erythema and induration. With the other strain, the average induration areas were as follows: T. phagedenis biotype English Reiter (34 mm^2), T. denticola (33.6 mm^2), T. phagedenis biotype Kazan 4 (27.9 mm^2), T. refringens biotype refringens (27.1 mm^2), T. phagedenis biotype Kazan 5 (27.1 mm^2), and T. scoliodontum (25.7 mm^2). Thus, T. refringens biotype Nichols exhibits the highest degree of sensitization while T. scoliodontum demonstrates the lowest degree of sensitization.

Comparison of the degree of skin reaction to different challenging antigens.

Table XIV summarized the means and standard errors of skin reaction of sensitized animals after the intradermal challenge with different skin test antigens. The mean of skin reactions of each challenging antigen was calculated from 168 readings (four replications from two related times, 24h and 48h, in three different days, 12, 20, and 30, respectively, (4 x 2 x 3), seven challenging antigens (7). It was found that T. refringens biotype Nichols induced the strongest skin reactions in sensitized animals by producing an average area of 40.7 mm^2 in erythema and induration. T. refringens biotype Nichols was the most potent skin test antigen. The findings of the other skin test antigens are as follows: T. denticola (37.5 mm^2), T. phagedenis biotype Kazan 5 (32.7 mm^2), and T. scoliodontum (23.1 mm^2). The saline was used for control and showed an average area of 7.7 mm^2 in erythema temporarily and subsided later.

TABLE XIV

MEANS AND STANDARD ERRORS OF THE DATA OBTAINED FROM HYPERSENSITIVIES TO A SINGLE CYTOPLASM IN GUINEA PIGS SENSITIZED WITH CYTOPLASMS OF DIFFERENT TREPONEMES

CHA	N	MEAN	S.E.SQRD	S.E.
1	168	0.231	0.000	0.012
2	168	0.346	0.000	0.012
3	168	0.327	0.000	0.012
4	168	0.354	0.000	0.012
5	168	0.375	0.000	0.012
6	168	0.407	0.000	0.012
7	168	0.391	0.000	0.012
8	168	0.077	0.000	0.012

CHA: challenging antigens
1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

Dermal hypersensitivity to challenging antigens.

The animals were immunized and skin tests were carried out after 12, 20, and 30 days of immunization. After 24, 48 and 72 hours of intradermal challenge, the skin reactions were measured. The degree of dermal reactivity was measured by two axis, longest and shortest of the area of erythema and induration to obtain an approximate of the area of the reaction by product of the two axes. The findings on the test of the individual strain are as follows:

(1) T. scoliodontum

The skin reaction of animals sensitized with T. scoliodontum and challenged on 12, 20 and 30 days, are shown in figure 2a, 2b, and 2c. The results were summarized as follows:

1) The skin tests performed on day 12 showed mild induration at 24h and all values were lesser than 40 mm^2. After 24h, all the reactions subsided. The homologous T. scoliodontum challenging antigen started with a reaction of 18.6 mm^2 at 24h and 10.6 mm^2 at 72h.

2) Skin challenged on day 20 showed greater erythema and induration. The skin reaction observed at 24h were greater than 48h and 72h. At 72h, most of the erythema and induration disappeared. It can be seen that T. scoliodontum challenging antigen induced the greatest erythema and induration in sensitizing animals by showing a reaction of 52.1 mm^2 at 24h and 27.9 mm^2 at 48h while T. phagedenis biotype Kazan 4 which started with a reaction of 46.3 mm^2 at 24h and 23.3 mm^2 at 48h, was the second potent challenging antigen. The findings of the other skin test antigens are of no significances.

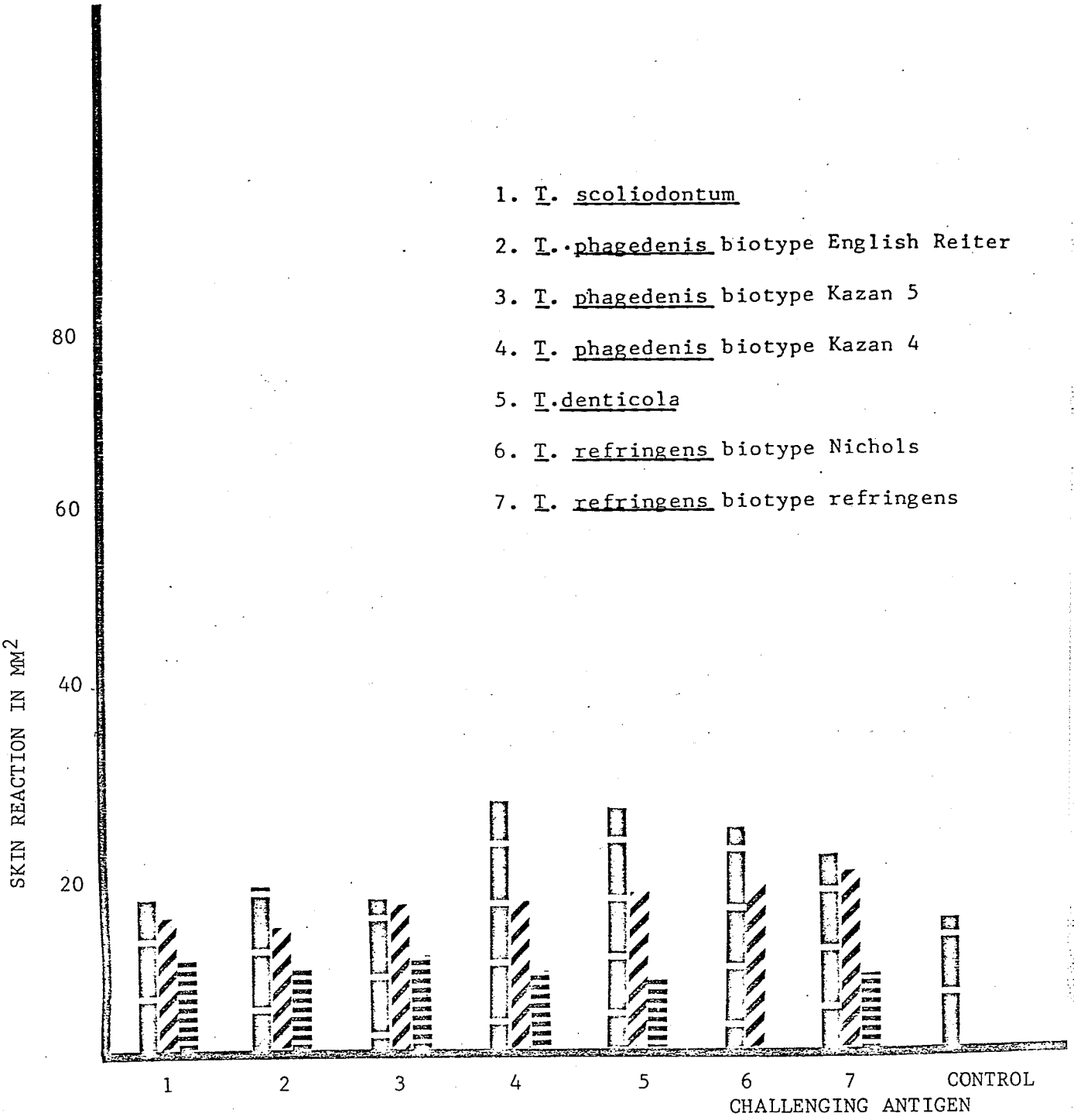


Fig.2a Skin reaction in mm^2 of guinea pigs sensitized with T. scoliodontum and skin-tested on 12 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

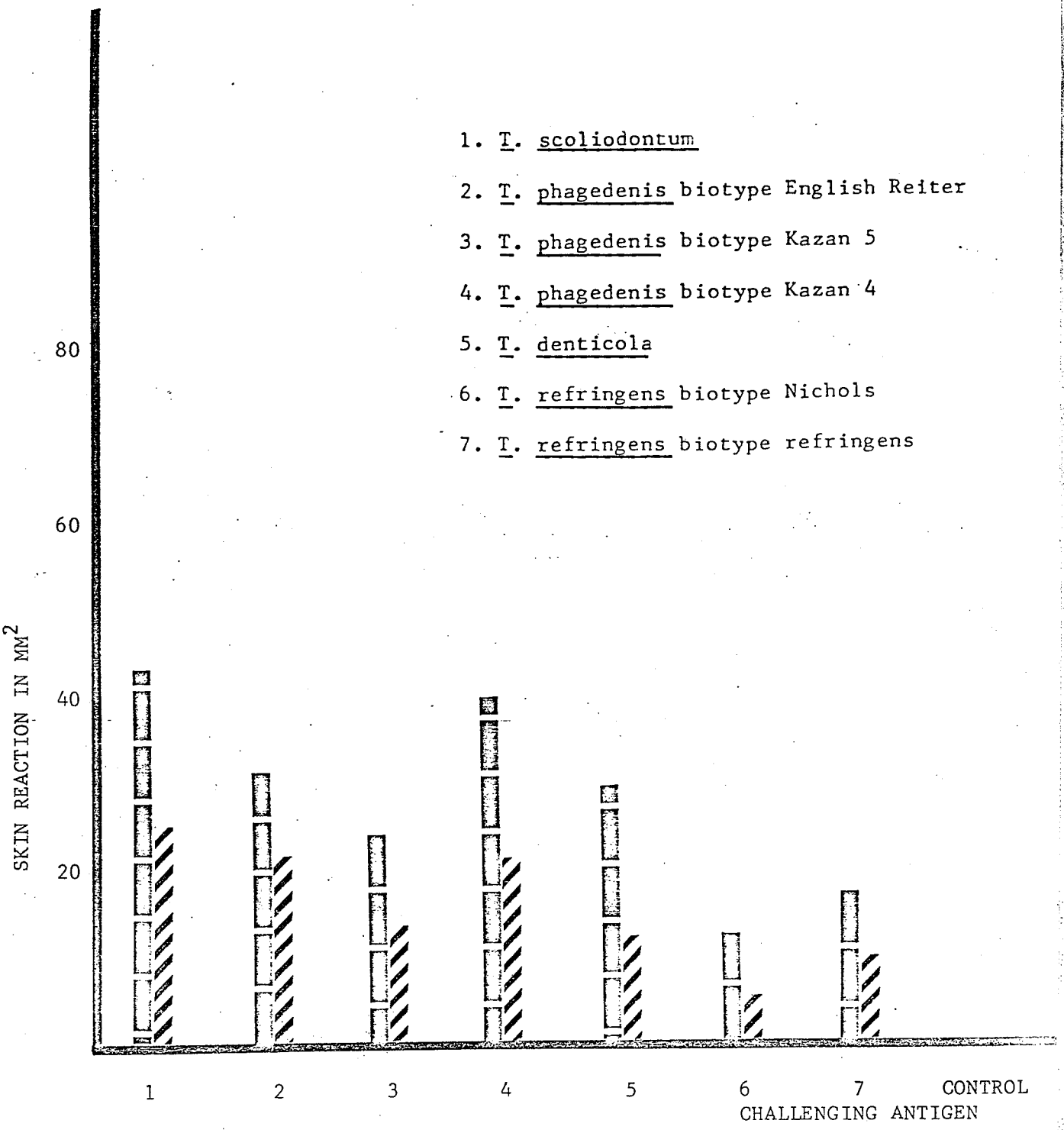


Fig.2b Skin reaction in mm^2 of guinea pigs sensitized with T.scoliodontum and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

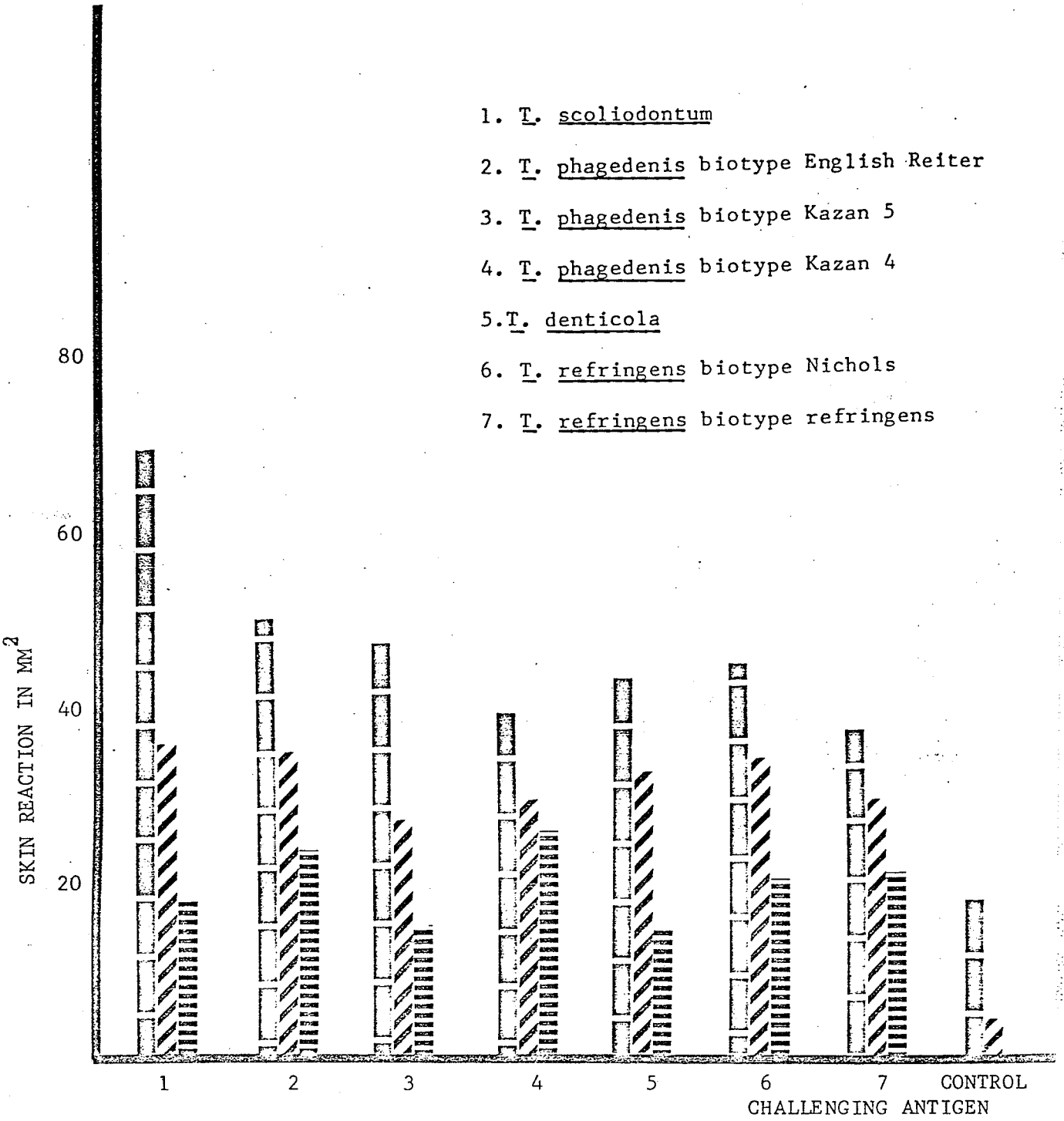


Fig. 2c Skin reaction in mm^2 of guinea pigs sensitized with T.scoliodontum and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.
measurements at 24h measurements at 48h measurements at 72h

3) The skin test on day 30 showed marked erythema and induration for only a few skin test antigens which gave a skin reaction area greater than 40 mm^2 at 24h. And again the reaction diminished at 48h and 72h. T. scoliodontum, T. phagedenis biotype English Reiter, T. phagedenis biotype Kazan 5, T. phagedenis biotype Kazan 4, T. denticola, T. refringens, and T. refringens biotype Nichols had skin indurations over 40 mm^2 at 24h. Still the homologous T. scoliodontum is the strongest challenging antigen among all.

4) The comparison of antigens used for intradermal challenge on animals sensitized with T. scoliodontum was shown on Table XV. The mean of skin reactions of each challenging antigen was calculated from 24 readings (four replications from two related times, 24h and 48h, of skin reactions in three different days, 12, 20, and 30, for skin challenge). The intensity of erythema and induration induced by different antigens are listed in decreasing order as follows: T. scoliodontum (38.9 mm^2), T. phagedenis biotype Kazan 4 (30.2 mm^2), T. phagedenis biotype English Reiter (29.4 mm^2), T. denticola (29.1 mm^2), T. phagedenis biotype Kazan 5 (25.1 mm^2), T. refringens biotype Nichols (24.9 mm^2), T. refringens biotype refringens (23.2 mm^2), and saline as the control (5.0 mm^2).

(II) T. phagedenis biotype English Reiter

Figure 3a, 3b, and 3c show the skin reactions of animals sensitized with T. phagedenis biotype English Reiter and subsequently challenged on 12, 20 and 30 days. The results are summarized as follows:

1) On the 12th day, the challenging antigen of T. phagedenis biotype English Reiter induced an area of 5 mm^2 erythema and induration at 24h

TABLE XV

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMALS SENSITIZED WITH T.SCOLIODONTUM

		N	MEAN	S.E.SQRD	S.E.
STR	CHA				
1	1	24	0.389	0.001	0.031
1	2	24	0.294	0.001	0.031
1	3	24	0.251	0.001	0.031
1	4	24	0.302	0.001	0.031
1	5	24	0.291	0.001	0.031
1	6	24	0.249	0.001	0.031
1	7	24	0.232	0.001	0.031
1	8	24	0.050	0.001	0.031

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQUD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

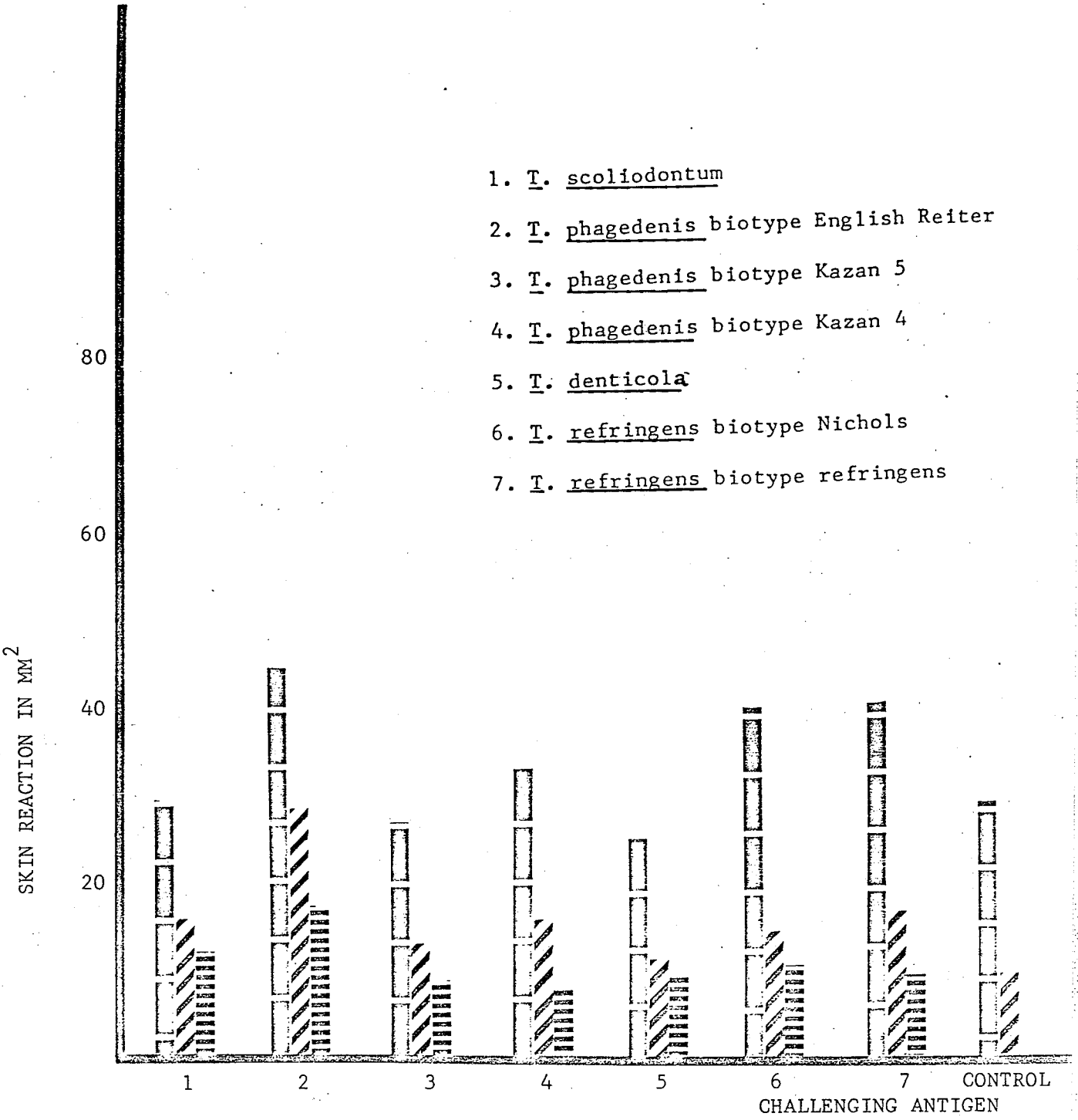


Fig 3a Skin reaction in mm^2 of guinea pigs sensitized with T.phagedenis biotype English Reiter and skin-testes on 12 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

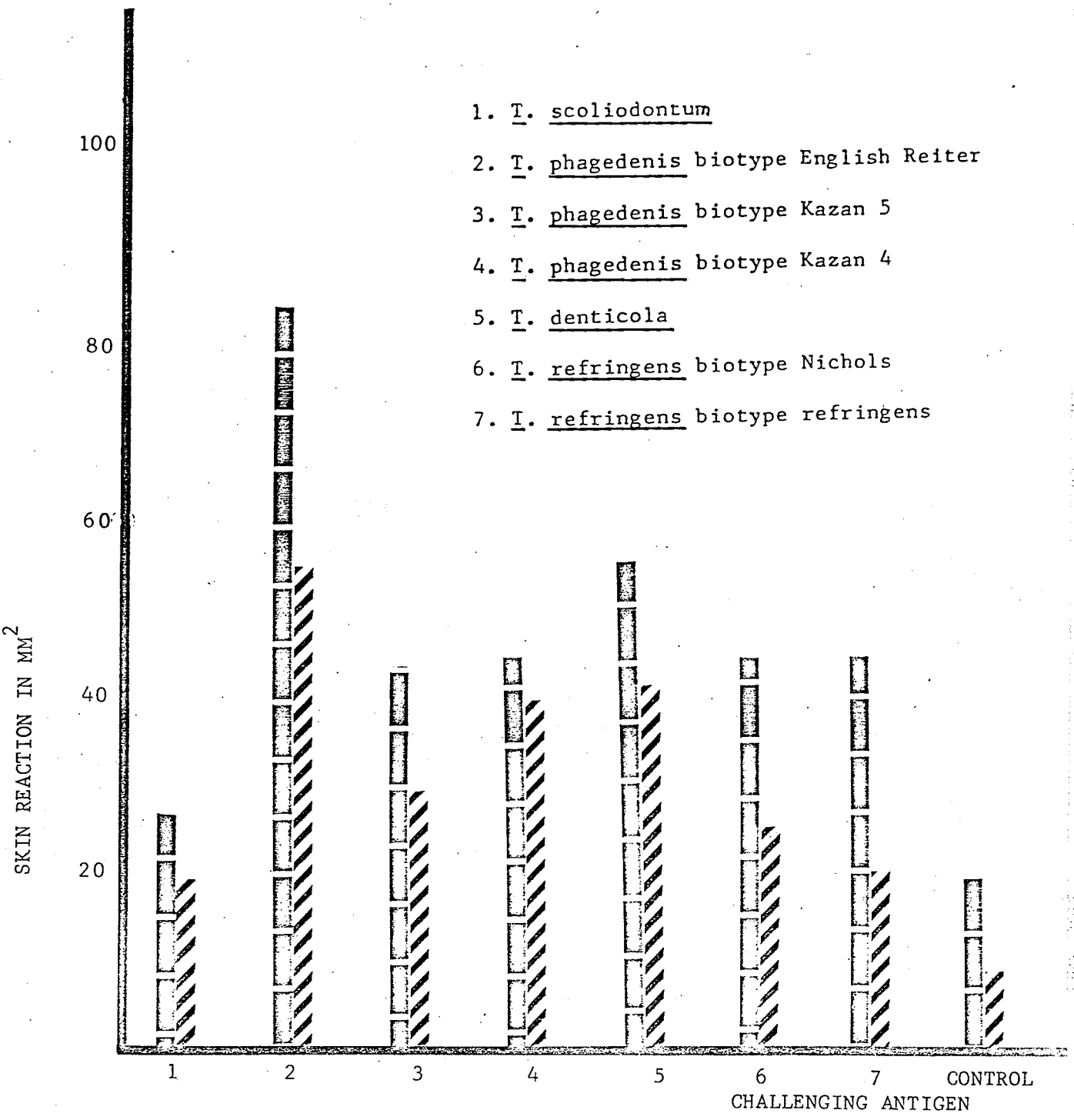


Fig.3b Skin reaction in mm^2 of guinea pigs sensitized with T.phagedenis biotype English Reiter and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurement at 48h measurements at 72h

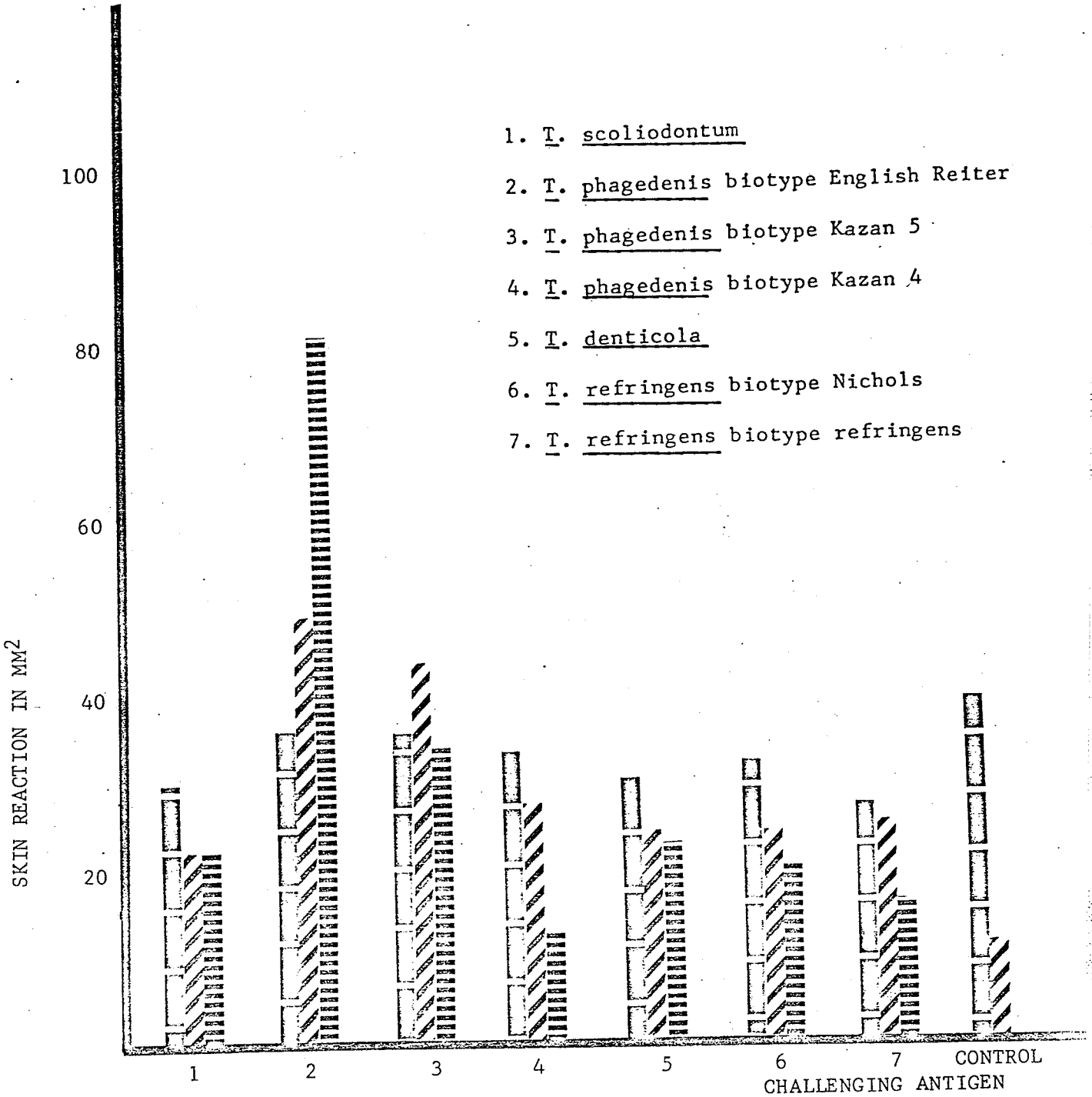


Fig.3c Skin reaction in mm^2 of guinea pigs sensitized with T. phagedenis biotype English Reiter and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

and 18.6 mm^2 at 72h; T. refringens biotype Nichols gave 46.1 mm^2 at 24h and 10 mm^2 at 72h; T. refringens biotype refringens gave 46.0 mm^2 at 24h, and 8.6 mm^2 at 72h. The other antigens showed insignificant indurations (less than 40 mm^2) at 24h and subsided at 48h and 72h.

2) All animals showed stronger erythema and induration when intradermally challenged on day 20. T. phagedenis biotype English Reiter, the challenging antigen showed marked erythema and induration of 99.5 mm^2 at 24h and 64.6 mm^2 at 48h; T. denticola was the second strongest challenging antigen in having a reaction of 63.3 mm^2 at 24h and 49 mm^2 at 48h. T. phagedenis biotype Kazan 5, T. phagedenis biotype Kazan 4, T. refringens biotype Nichols and T. refringens biotype refringens had the skin indurations of over 40 mm^2 at 24h, and the induration abated at 48h. T. scoliodontum and saline as the control showed mild indurations (less than 40 mm^2) on the 20 day intradermal challenge.

3) On the 30th day intradermal challenge, T. phagedenis biotype English Reiter showed a peaked delayed reaction at 72h (93.2 mm^2) compared with 24h (39.6 mm^2) and 48h (56.3 mm^2). T. phagedenis biotype Kazan 5 had a peaked delayed reaction at 48h (49.5 mm^2) compared with 24h (41.0 mm^2) and 72h (38.5 mm^2). The other antigens did not show significant induration (less than 40 mm^2) at 24h, 48h and 72h.

4) The results of all skin tests from each challenging antigen were summarized in Table XVI. It has been shown that T. phagedenis biotype English Reiter, the strongest challenging antigen elicited an average of 58.7 mm^2 induration. T. phagedenis biotype Kazan 5, and second challenging antigen, evoked an average of 36.3 mm^2 induration. The effects of the other

TABLE XVI.

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMALS SENSITIZED WITH T.PHAGEDENIS BIOTYPE ENGLISH REITER

STR	CHA	N	MEAN	S.E.SQRD	S.E.
2	1	24	0.280	0.001	0.031
2	2	24	0.587	0.001	0.031
2	3	24	0.363	0.001	0.031
2	4	24	0.353	0.001	0.031
2	5	24	0.340	0.001	0.031
2	6	24	0.322	0.001	0.031
2	7	24	0.316	0.001	0.031
2	8	24	0.161	0.001	0.031

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQUD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

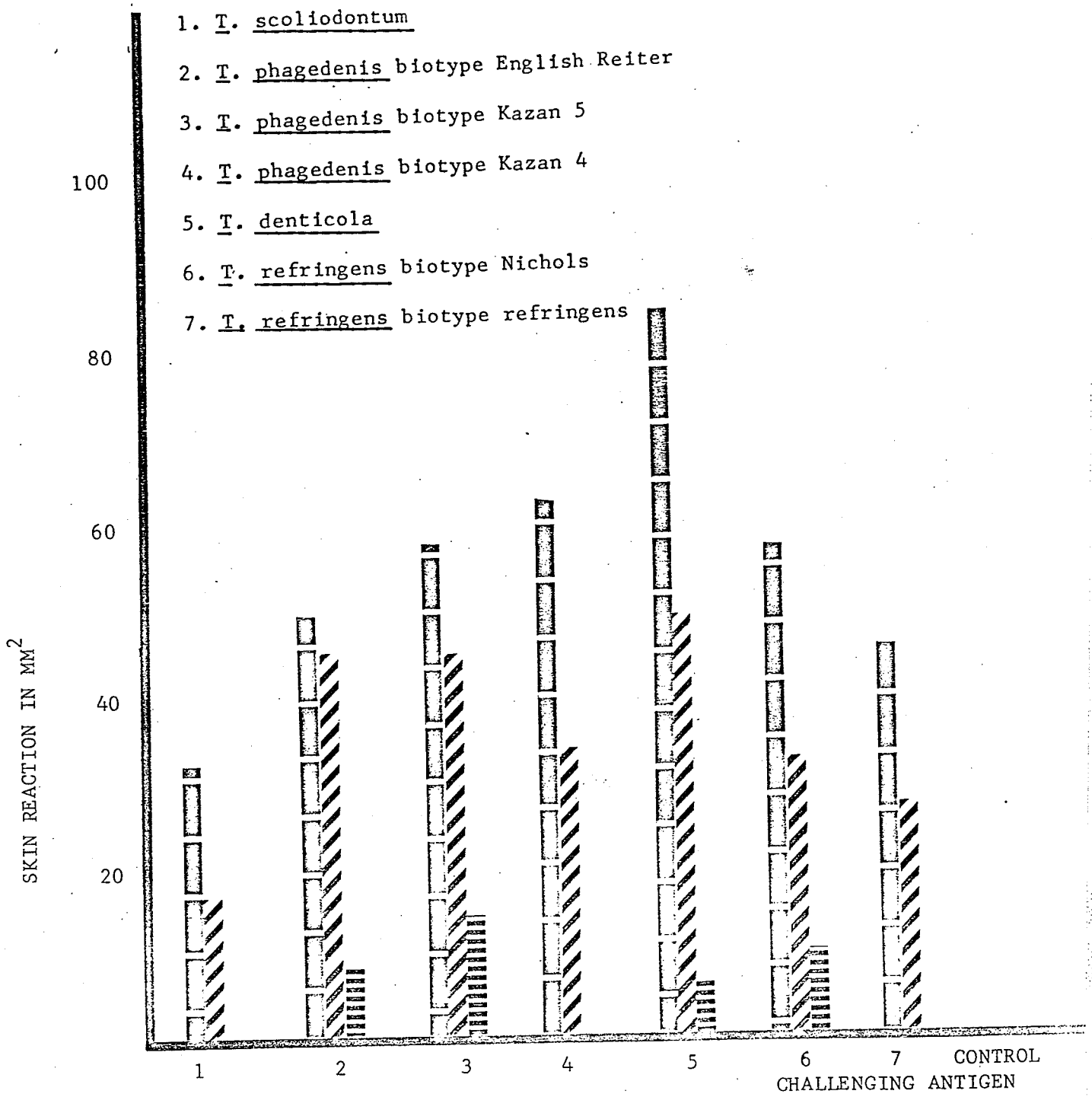


Fig.4a Skin reaction in mm^2 of guinea pigs sensitized with T.phagedenis biotype Kazan 5 and skin-tested on 12 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

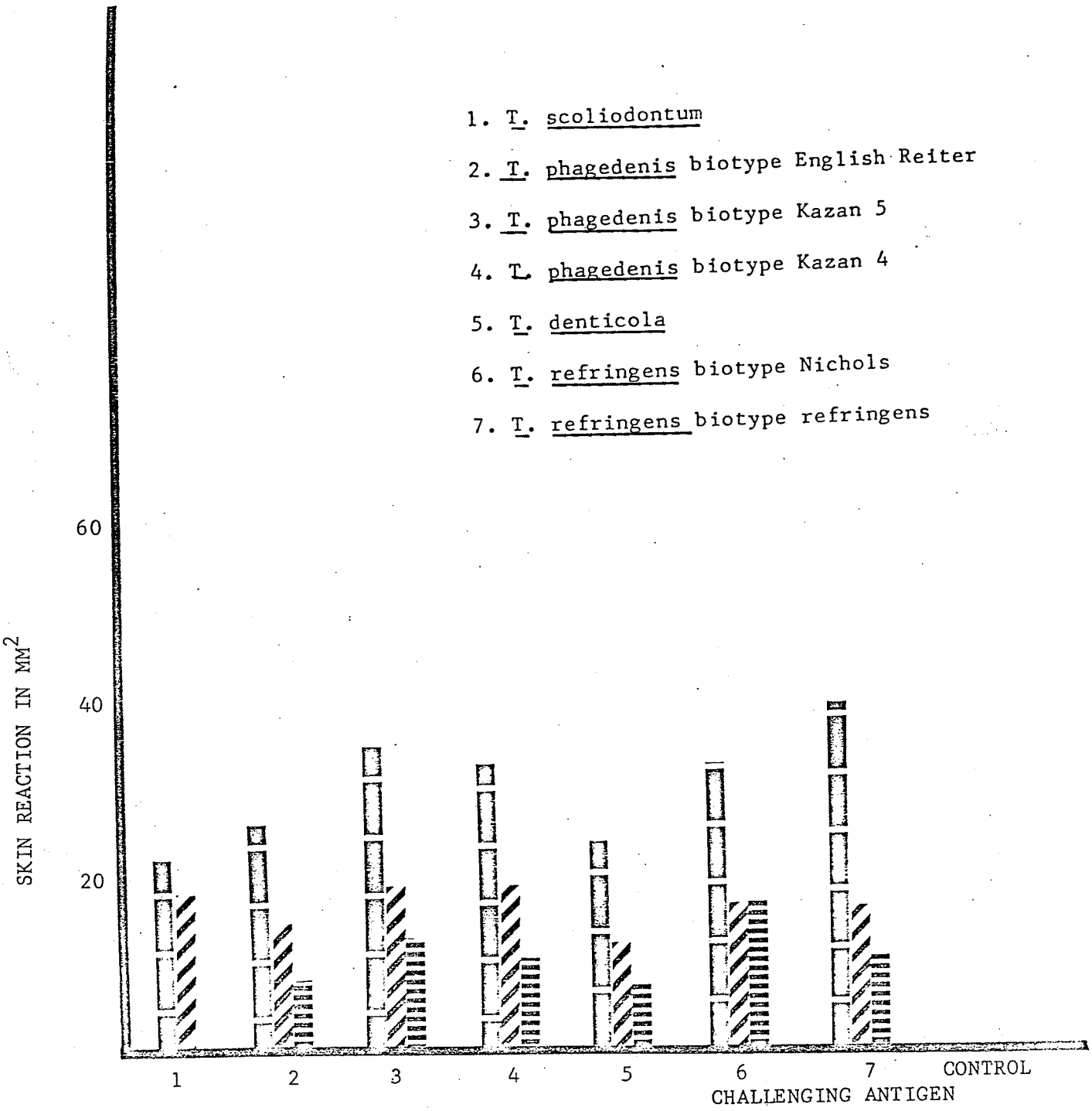


Fig.4b Skin reaction in mm^2 of guinea pigs sensitized with T.phagedenis biotype Kazan 5 and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

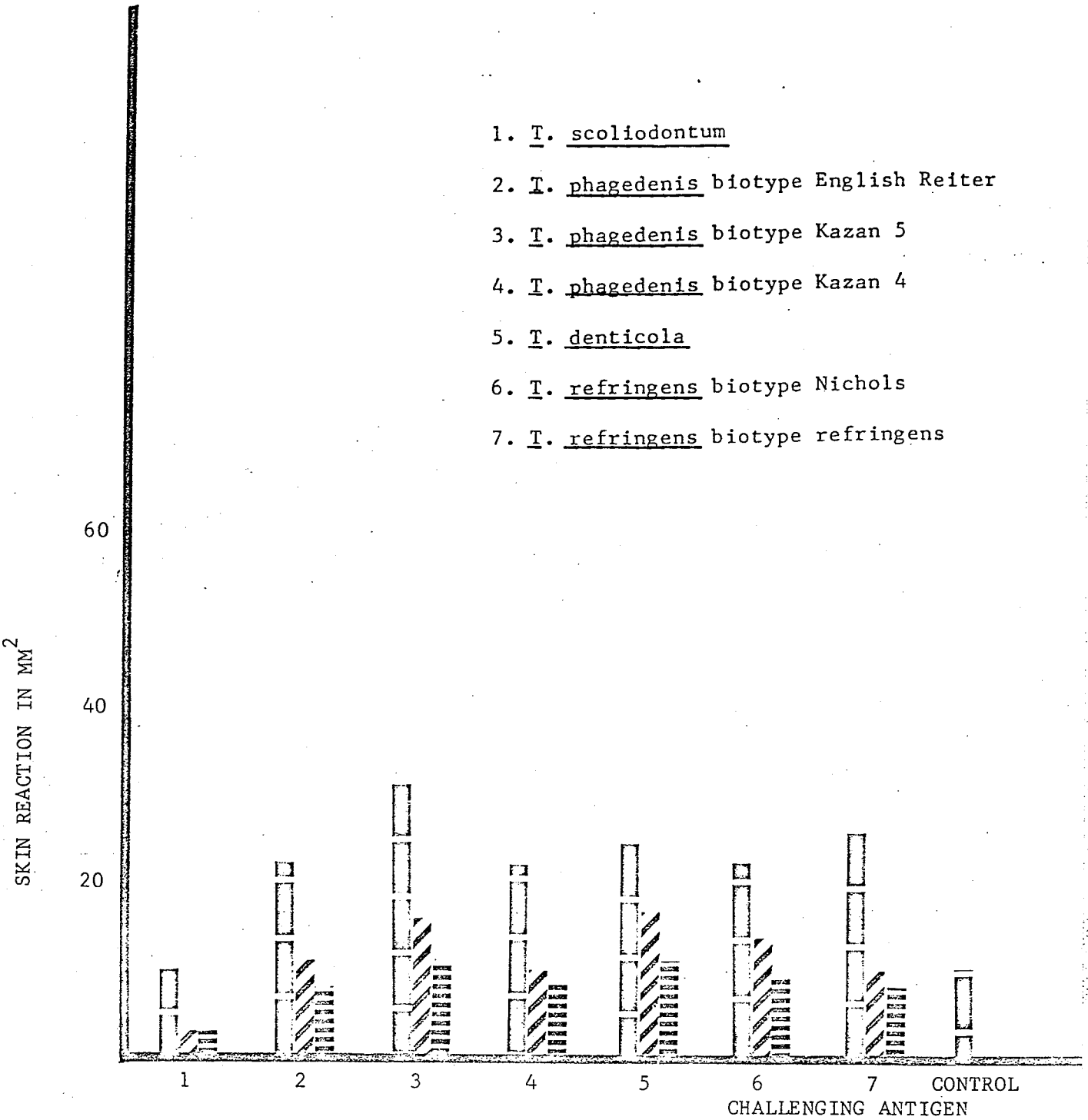


Fig.4c Skin reaction in mm^2 of guinea pigs sensitized with T. phagedenis biotype Kazan 5 and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

skin test antigens are as follows: T. scoliodontum (28.0 mm^2), T. phagedenis biotype Kazan 4 (35.3 mm^2), T. denticola (34.0 mm^2), T. refringens biotype Nichols (32.2 mm^2) and T. refringens biotype refringens (31.6 mm^2). The saline was used as the control and showed temporal erythema of 16 mm^2 without induration.

(III) T. phagedenis biotype Kazan 5

Figure 4a, 4b, and 4c describe the skin reactions of animals sensitized with T. phagedenis biotype Kazan 5 and challenged on 12, 20, and 30 days after sensitization.

1) The intradermal challenge on day 12 showed stronger reactions than day 20 and day 30. The skin induration at 24h are the following: T. phagedenis biotype English Reiter (55.0 mm^2), T. phagedenis biotype Kazan 5 (65.0 mm^2), T. phagedenis biotype Kazan 4 (71.0 mm^2), T. denticola (96.0 mm^2), T. refringens biotype Nichols (50.0 mm^2), T. refringens biotype refringens (50.0 mm^2), and T. scoliodontum (35.0 mm^2). The induration of saline was negative at 24h. At 48h, only T. phagedenis biotype English Reiter, T. phagedenis biotype Kazan 5, T. phagedenis biotype Kazan 4, and T. denticola still effected an induration of over 40 mm^2. The difference of indurations between 24h and 48h of T. phagedenis biotype English Reiter is 5 mm^2 which is the smallest difference among the challenging antigens. Although T. denticola gave rise to the strongest induration at 24h (96.0 mm^2), it subsided very fast and had an induration of 54 mm^2 at 48h. The differences of induration between 24h and 48h of other challenging antigens are as follows: T. phagedenis biotype Kazan 4 (29.0 mm^2), T. refringens biotype Nichols (28.0 mm^2), T. refringens biotype refringens (21.0 mm^2), and T. scoliodontum (17.0 mm^2).

TABLE XVII

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMAL SENSITIZED WITH T.PHAGEDENIS BIOTYPE KAZAN 5

STR	CHA	N	MEAN	S.E.SQRD	S.E.
3	1	24	0.148	0.001	0.031
3	2	24	0.305	0.001	0.031
3	3	24	0.377	0.001	0.031
3	4	24	0.327	0.001	0.031
3	5	24	0.375	0.001	0.031
3	6	24	0.322	0.001	0.031
3	7	24	0.290	0.001	0.031
3	8	24	0.025	0.001	0.031

STR: strain for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQUD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

2) Intradermal challenge of day 20 showed mild induration for all the antigens. Only T. refringens biotype refringens produced an induration of 43.5 mm^2 at 24h and subsided to 18.8 mm^2 at 48h. The induration of other antigens were less than 40 mm^2 and even lesser at 48h.

3) Intradermal challenge on day 30 was insignificant and all measurements of induration were less than 40 mm^2 at 24h and 48h.

4) The averages of induration induced by each challenging antigen were listed in Table XVII and the results are as follows: T. scoliodontum (14.8 mm^2) T. phagedenis biotype English Reiter (30.5 mm^2), T. phagedenis biotype Kazan 5 (37.7 mm^2), T. phagedenis biotype Kazan 4 (32.7 mm^2), T. denticola (37.5 mm^2), T. refringens biotype Nichols (32.2 mm^2), T. refringens biotype refringens (29.0 mm^2) and saline (2.5 mm^2). The homologous challenging antigen of T. phagedenis is biotype Kazan 5 still induced the strongest reaction among the challenging antigens.

(IV) T. phagedenis biotype Kazan 4

The skin reactions of animals sensitized with T. phagedenis biotype Kazan 4 and challenged on 12, 20, and 30 days, are shown in figures 5a, 5b, and 5c. The results were as follows:

1) The skin tests performed on day 12 were insignificant. Only the homologous challenging antigen, T. phagedenis biotype Kazan 4, elicited an induration of 46.4 mm^2 at 24h. The induration of the other challenging antigens were less than 40 mm^2 at 24h and subsided at 48h and 72h.

2) Skin challenged on day 20 showed the strongest erythema and induration. Most of the antigens used for intradermal challenge indueed skin indurations over 40 mm^2 at 24h. These antigens were T. phagedenis biotype English Reiter (43.6 mm^2), T. phagedenis biotype Kazan 5 (51.6 mm^2), T. phagedenis

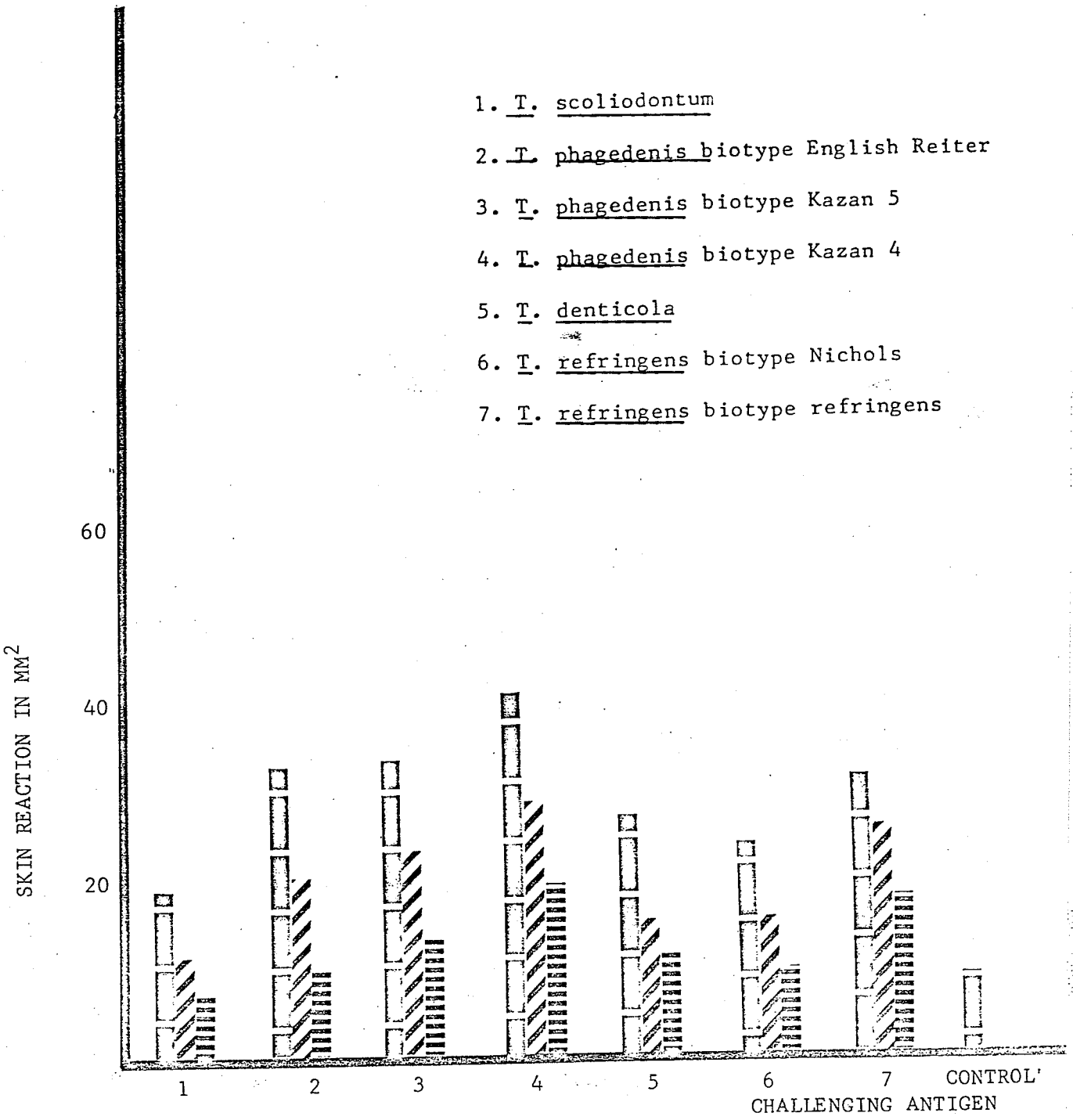


Fig.5a Skin reaction in mm^2 of guinea pig sensitized with T.phagedenis biotype Kazan 4 and skin-tested on 12 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurments at 24h measurements at 48h measurements at 72h

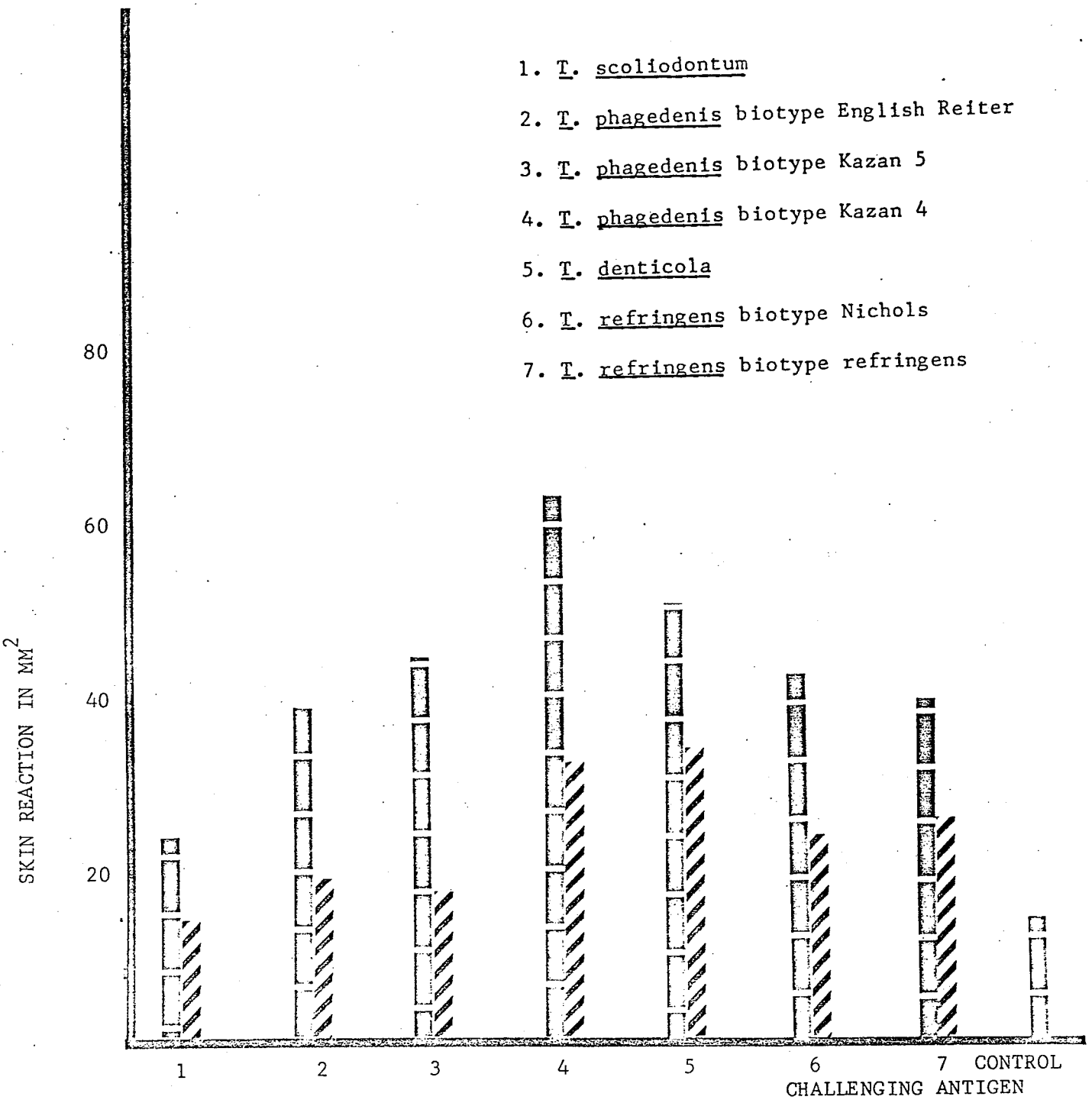


Fig.5b Skin reaction in mm^2 of guinea pig sensitized with T.phagedenis biotype Kazan 4 and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

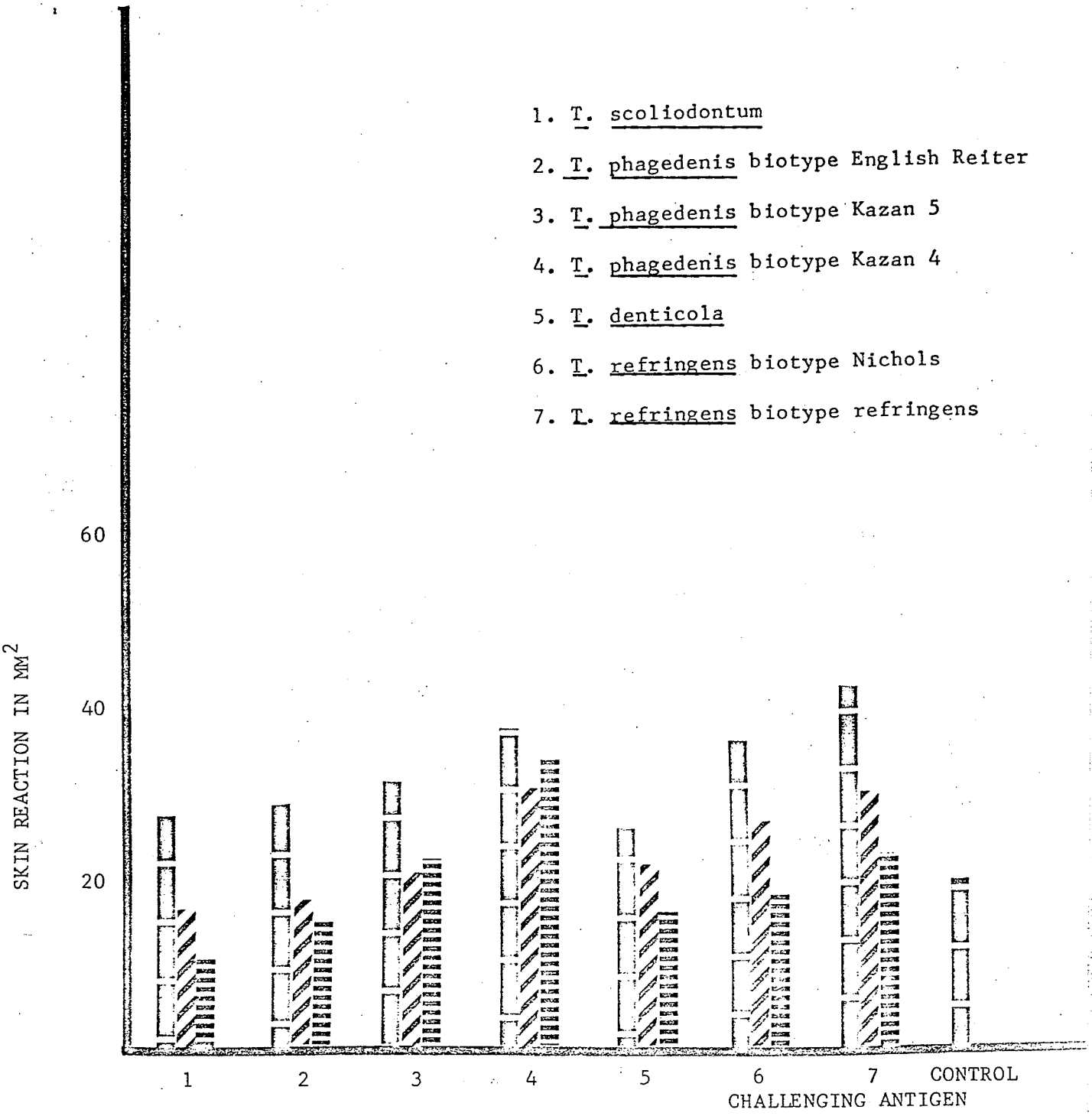


Fig. 5c Skin reaction in mm^2 of guinea pigs sensitized with T.phagedenis biotype Kazan 4 and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

biotype Kazan 4 (72.0 mm^2), T. denticola (57.0 mm^2), T. refringens biotype Nichols (47.5 mm^2), and T. refringens (46.6 mm^2). At 48h, all the indurations subsided to an area less than 40 mm^2 as listed below: T. phagedenis biotype English Reiter (20.0 mm^2), T. phagedenis biotype Kazan 5 (19.2 mm^2), the homologous T. phagedenis biotype Kazan 4 (35.5 mm^2), T. denticola (36.7 mm^2), T. refringens biotype Nichols (26.2 mm^2), and T. refringens biotype refringens (29.2 mm^2). T. scoliodontum and saline induced low indurations of 26.0 mm^2 and 12.0 mm^2, respectively, at 24h. The homologous challenging antigen, T. phagedenis biotype Kazan 4, induced the strongest induration at 24h (72.0 mm^2) and remained a mild induration at 48h (35.5 mm^2).

3) Only a few challenging antigens induced a skin induration area greater than 40 mm^2 on the 30th day intradermal challenge. T. phagedenis biotype Kazan 4 provoked an induration of 41.0 mm^2 at 24h and 32.9 mm^2 at 48h. At 72h, the induration was 37.5 mm^2. T. refringens biotype refringens, the next strongest challenging antigen, induced an induration of 46.0 mm^2 (greater than 40 mm^2) at 24h, 32.5 mm^2 at 48h, and 23.8 mm^2 at 72h. The skin indurations of other antigens were of no significance at 24h and 48h.

4) The skin reactions induced by different challenging antigens were summarized in Table XVIII. It was found that T. phagedenis biotype Kazan 4 induced the greatest induration of 42.4 mm^2 in average. T. refringens biotype refringens, the second potent antigen, provoked an induration of 36.5 mm^2 in average. The results of the other antigens were 19.7 mm^2 (T. scoliodontum), 28.1 mm^2 (T. phagedenis biotype English

TABLE XVIII

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMAL SENSITIZED WITH T.PHAGEDENIS BIOTYPE KAZAN 4

STR	CHA	N	MEAN	S.E.SQRD	S.E.
4	1	24	0.197	0.001	0.031
4	2	24	0.281	0.001	0.031
4	3	24	0.298	0.001	0.031
4	4	24	0.424	0.001	0.031
4	5	24	0.301	0.001	0.031
4	6	24	0.317	0.001	0.031
4	7	24	0.365	0.001	0.031
4	8	24	0.048	0.001	0.031

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQUD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

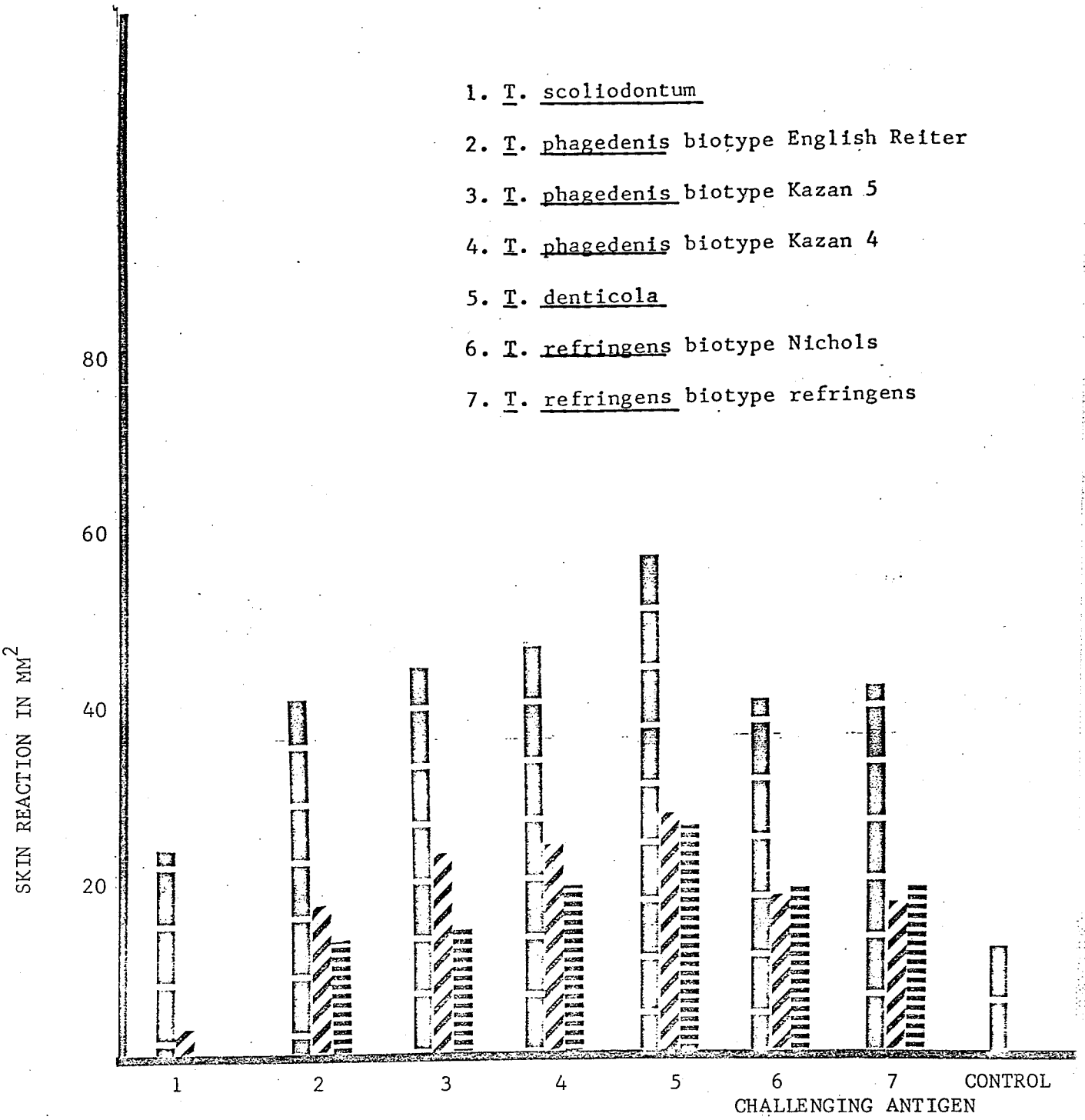


Fig.6a Skin reaction in mm^2 of guinea pigs sensitized with T.denticola and skin-tested on 12 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

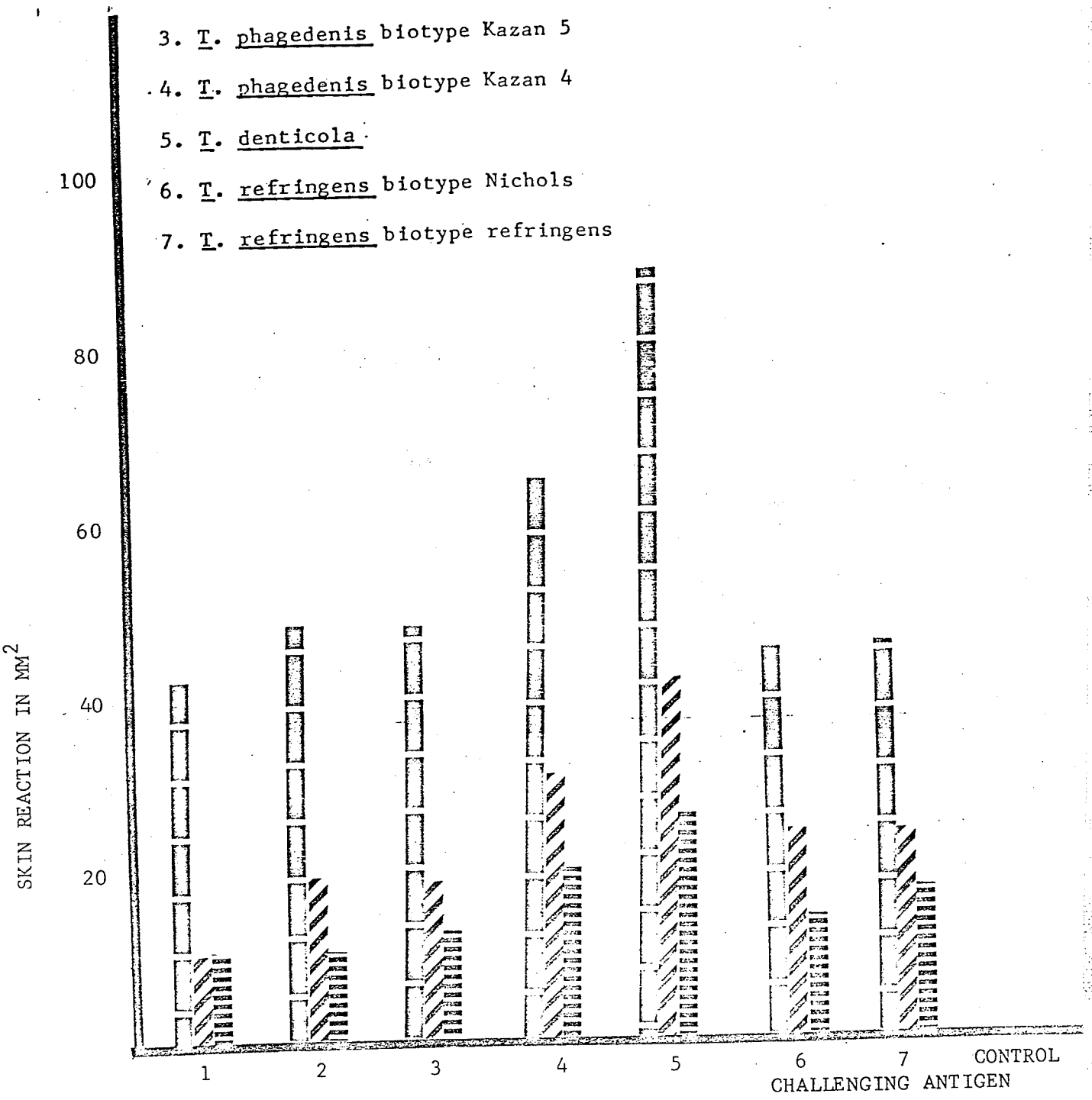


Fig.6b Skin reaction in mm^2 of guinea pigs sensitized with T.denticola and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

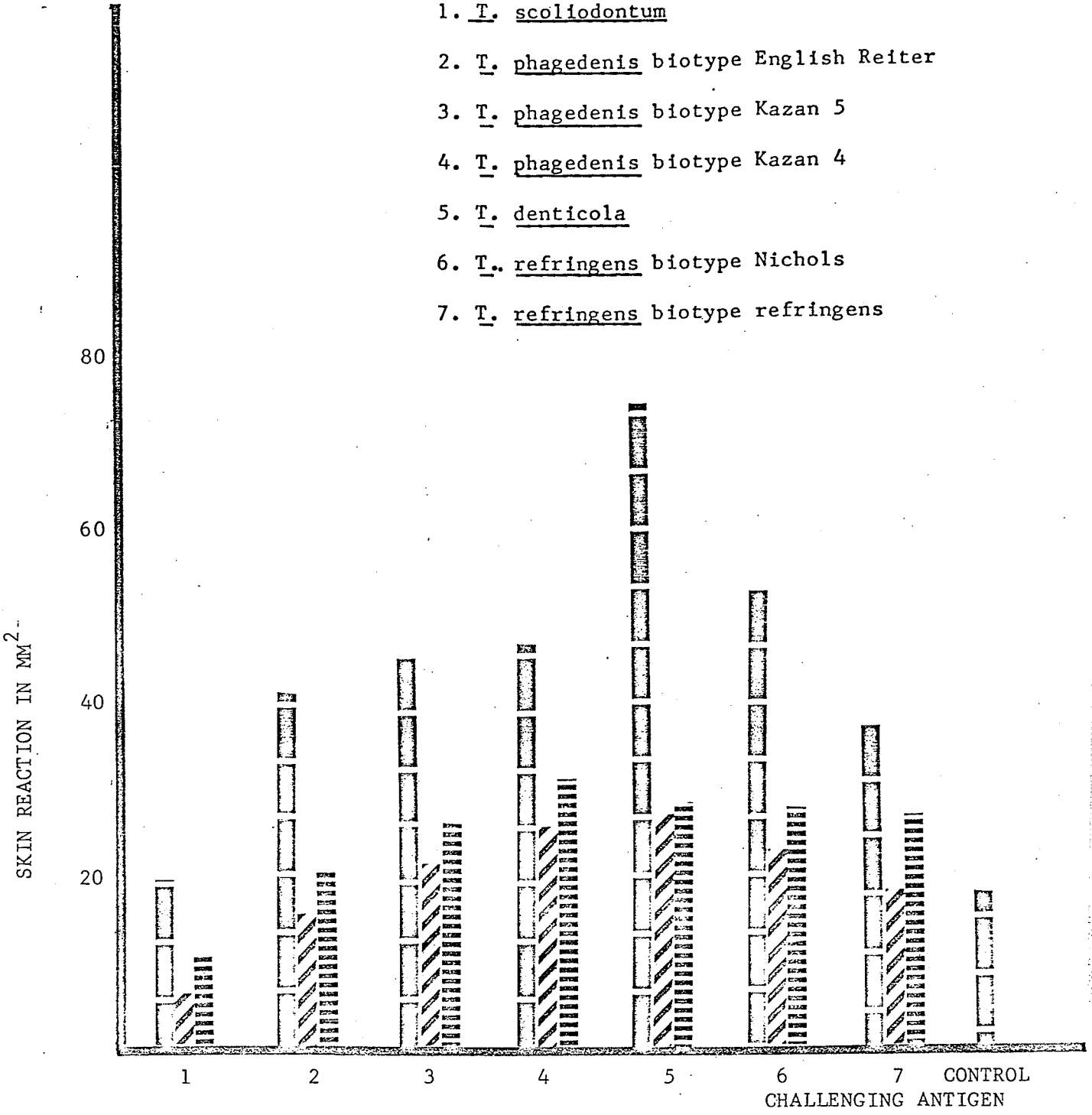


Fig.6c Skin reaction in mm^2 of guinea pigs sensitized with T.denticola and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

Reiter), 29.8 mm^2 (T. phagedenis biotype Kazan 5), 30.1 mm^2 (T. denticola), 37.1 mm^2 (T. refringens biotype refringens), and 4.8 mm^2 for saline.

(V) T. denticola

Figures 6a, 6b, and 6c showed the skin reactions of animals sensitized with T. denticola and challenged on 12, 20 and 30 days after sensitization. The results were as follows:

1) Most of the challenging antigens started with a marked erythema and indurations at 24h on day 12 intradermal challenge. These antigens were T. phagedenis biotype English Reiter (45.0 mm^2), T. phagedenis biotype Kazan 5 (52.0 mm^2), T. phagedenis biotype Kazan 4 (51.0 mm^2), T. denticola, the homologous antigen, 63.0 mm^2, T. refringens biotype Nichols (44.0 mm^2), and T. refringens biotype refringens (47.0 mm^2). T. scoliodontum and saline gave rise to very low indurations at 24h. At 48h, all the indurations subsided and the readings were T. phagedenis biotype Kazan 5 (25.0 mm^2), T. phagedenis biotype Kazan 4 (26.0 mm^2), T. denticola (30.0 mm^2), T. refringens biotype Nichols (20.0 mm^2), T. refringens biotype refringens (18.0 mm^2). Although the homologous T. denticola antigen induced the strongest induration subsided to less than 40 mm^2 at 48h.

2) All challenging antigens induced indurations over 40 mm^2 at 24h on day 20 intradermal challenge. The results at 24h were T. scoliodontum (47.0 mm^2), T. phagedenis biotype English Reiter (54.0 mm^2), T. phagedenis biotype Kazan 5 (55.o mm^2), T. phagedenis biotype Kazan 4 (71.0 mm^2), T. denticola (93.0 mm^2), T. refringens biotype Nichols (50.0 mm^2), and

T. refringens biotype refringens (50.0 mm^2). Saline used as the control was negative. At 48h, all the induration except the one induced by T. denticola (47.0 mm^2), subsided to non-significance. T. denticola, the homologous antigen was the most potent challenging antigen in the 20th day skin test.

3) On the 30th day skin test, the skin reactions were marked erythema and induration at 24h, subsided at 48h and interestingly increased at 72h. The results of the indurations were as follows: T. scoliodontum started with an induration of 21.0 mm^2 at 24h, 6 mm^2 at 48h, and 11 mm^2 at 72h. T. phagedenis biotype English Reiter began with an induration of 47.0 mm^2 at 24h, 17.0 mm^2 at 48h, and 22.0 mm^2 at 72h. T. phagedenis biotype Kazan 5 had an induration of 50.0 mm^2 at 24h, 23.0 mm^2 at 48h, and 23.0 mm^2 at 72h. T. phagedenis biotype Kazan 4 gave rise to an induration of 50.0 mm^2 at 24h, 23.0 mm^2 at 48h, and 23,0 mm^2 at 72h. The homologous antigen, T. denticola started with an induration of 83.0 mm^2 at 24h, 33.0 mm^2 at 48h, and 34.0 mm^2 at 72h. T. refringens biotype Nichols began with 60.0 mm^2 at 24h, 24.0 mm^2 at 48h, and 32.0 mm^2 at 72h. Finally T. refringens biotype refringens induced an induration of 42.0 mm^2 at 24h, 20.0 mm^2 at 48h, and 30.0 mm^2 at 72h. Saline produced an induration of 20 mm^2 at 24h and was zero at 48h and 72h.

4) The results of all the reactions were pooled, and the averages of induration induced by each antigen (see Table XIX) were calculated. It can be seen that T. denticola, the most potent antigen, elicited an average of 57.9 mm^2. The average induced by the other challenging

TABLE XIX

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMALS SENSITIZED WITH T. DENTICOLA

		N	MEAN	S.E.SQRD	S.E.
STR	CHA				
5	1	24	0.195	0.001	0.031
5	2	24	0.370	0.001	0.031
5	3	24	0.373	0.001	0.031
5	4	24	0.579	0.001	0.031
5	5	24	0.403	0.001	0.031
5	6	24	0.378	0.001	0.031
5	7	24	0.337	0.001	0.031
5	8	24	0.052	0.001	0.031

STR: strains for sensitization

GHA: challegning antigens

N: number of readings

S.E.SUID: standard error of square

S.E.: standard error

1. T. scoliodontum
2. T. phagedenis biotype English Reiter
3. T. phagedenis biotype Kazan 5
4. T. phagedenis biotype Kazan 4
5. T. denticola
6. T. refringens biotype Nichols
7. T. refringens biptype refringens
8. saline as the control

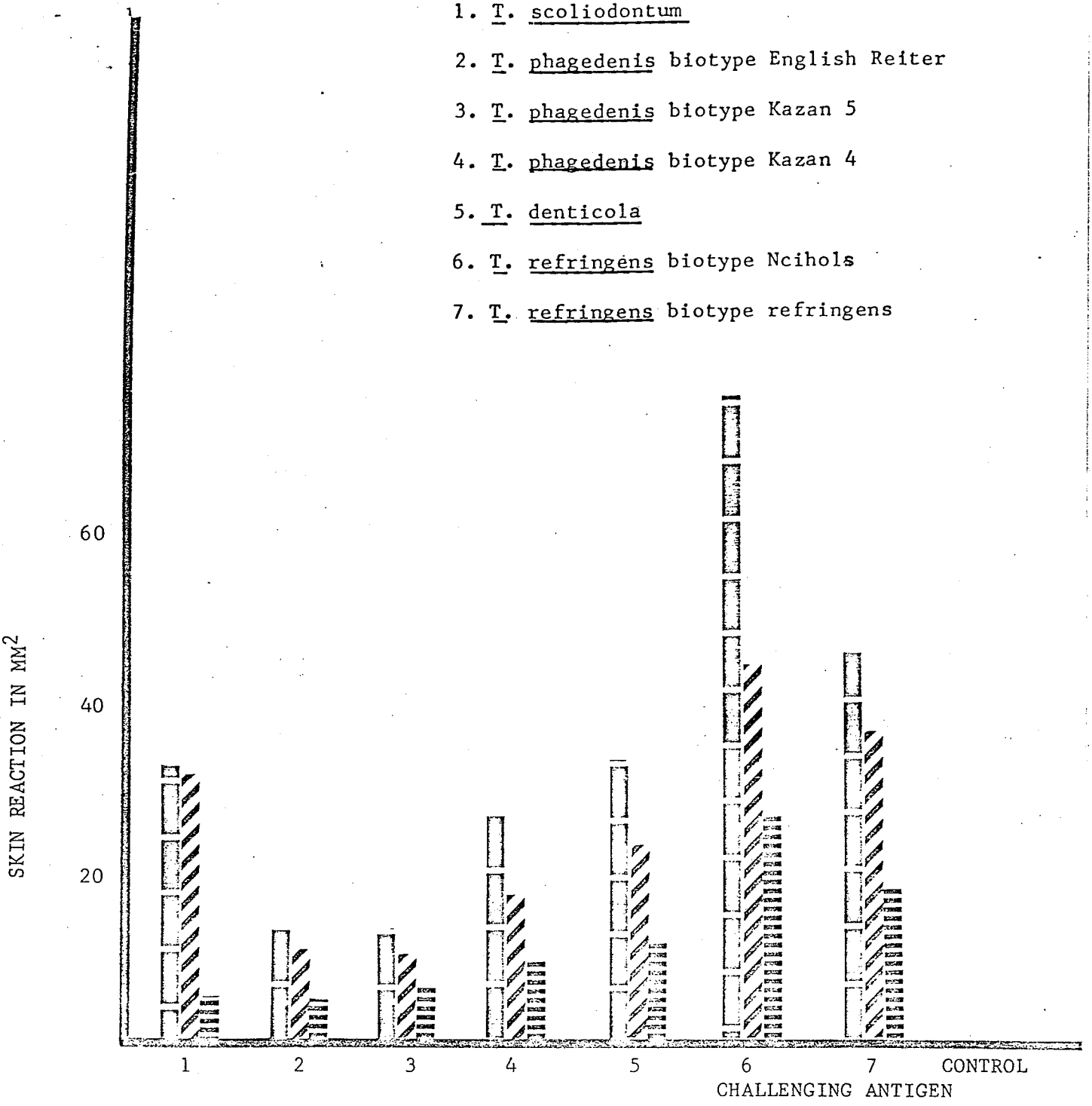


Fig.7a Skin reaction in mm^2 of guinea pigs sensitized with T.refringens biotype Nichols and skin-tested on 12 days with challenging antigens. Each vertical bar represents averqge values for four guinea pigs.

measurements at 24h measurements at 48h measurement at 72h

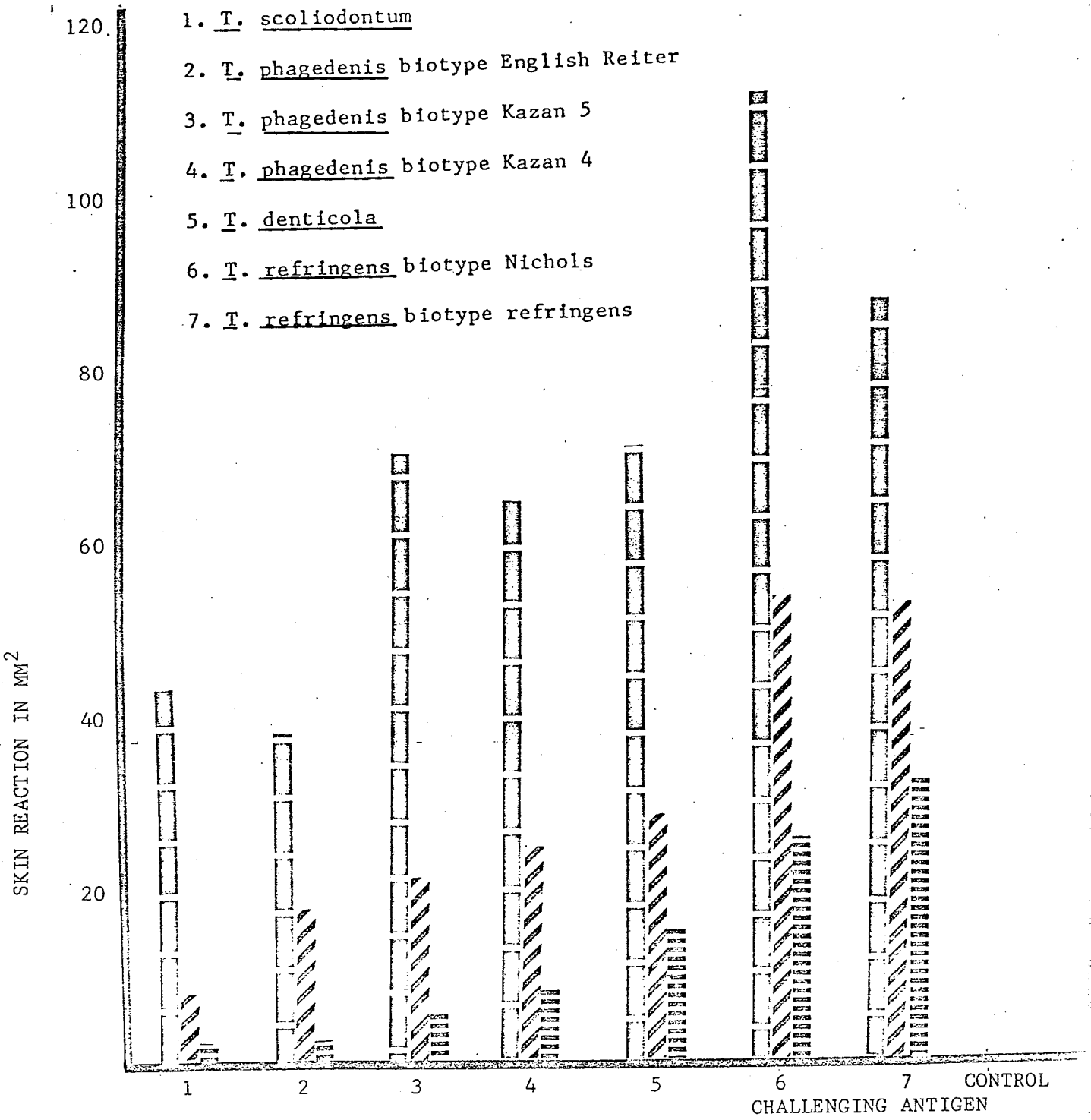


Fig.7b Skin reaction in mm^2 of guinea pigs sensitized with T. refringens biotype Nichols and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

antigens were T. scoliodontum (19.5 mm^2), T. phagedenis biotype English Reiter (37.0 mm^2), T. phagedenis biotype Kazan 5 (37.3 mm^2), T. phagedenis biotype Kazan 4 (40.5 mm^2), T. refringens biotype Nichols (37.8 mm^2), T. refringens biotype refringens (33.7 mm^2), and saline as the control (5.2 mm^2).

(VI) T. refringens biotype Nichols

The animals sensitized with T. refringens biotype Nichols were skin challenged on day 12, 20, and 30 after the sensitization.

1) It can be seen in figures 7a, the day 12 skin challenge, the homologous T. refringens biotype Nichols and T. refringens biotype refringens induced marked erythema and induration at 24h and the indruations remained moderately intensive at 48h. The indurations of other challenging antigens were insignificant (less than 40 mm^2). At 24h, T. refringens biotype Nichols provoked an induration of 86.0 mm^2. The induration subsided to 47.6 mm^2 at 48h and 29.3 mm^2 at 72h. T. refringens biotype refringens had an induration of 52.7 mm^2 at 24h, which subsided to 40.7 mm^2 48h and 19.8 mm^2 at 72h. The induration of saline was negative.

2) Figure 7b showed the skin reactions of the 20th day skin challenge. All antigens induced indurations over 40 mm^2 at 24h, while only T. refringens biotype Nichols and T. refringens biotype refringens still evoked an induration over 40 mm^2 at 48h. The results at 24h were T. scoliodontum (49.7 mm^2), T. phagedenis biotype English Reiter (43.0 mm^2), T. phagedenis biotype Kazan 5 (78.1 mm^2), T. phagedenis biotype Kazan 4 (74.0 mm^2), T. denticola (80.1 mm^2), T. refringens biotype Nichols (128.7 mm^2), and T. refringens biotype refringens (105.0 mm^2). The homologous antigen,

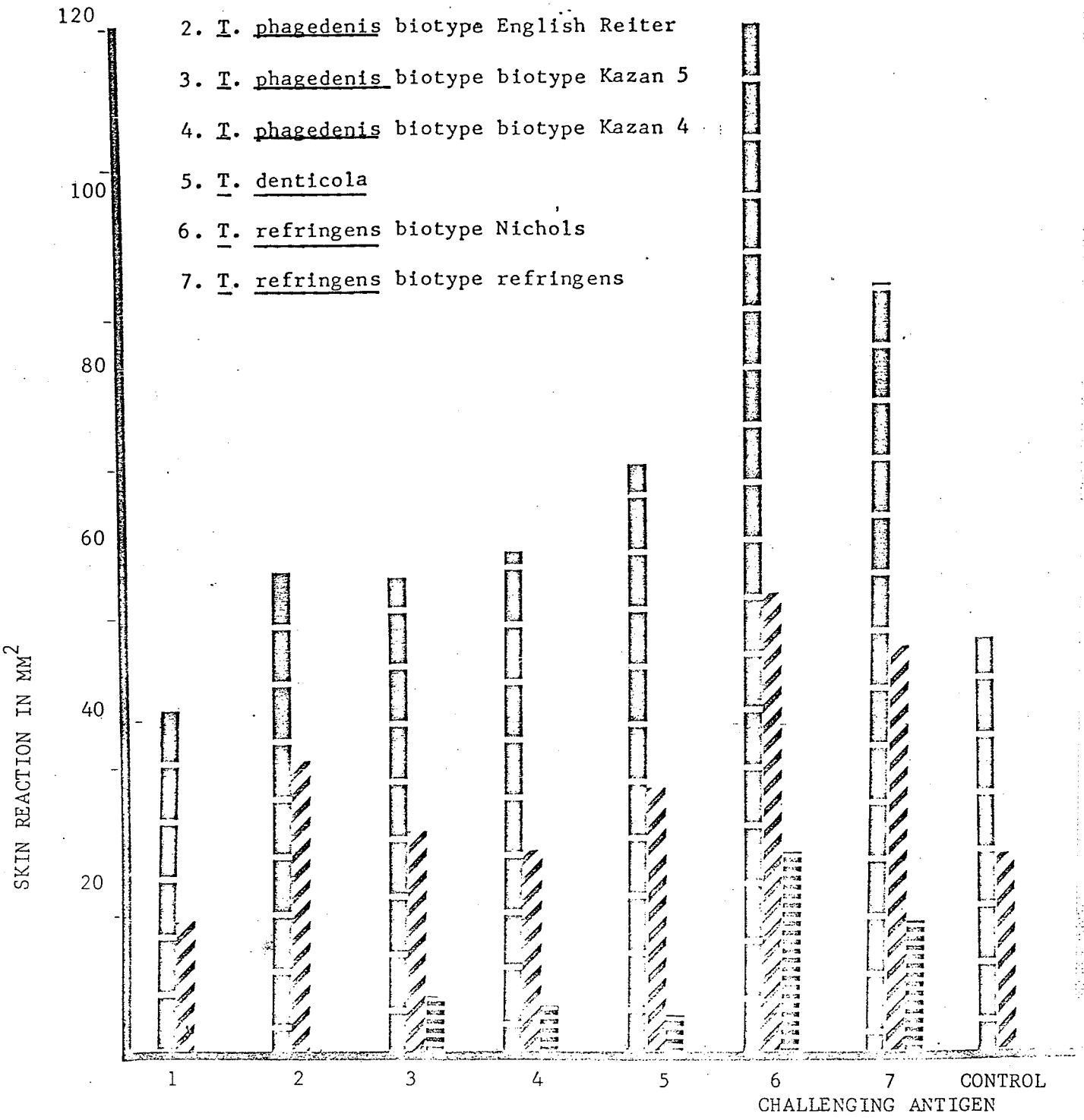


Fig.7c Skin reaction in mm^2 of guinea pigs sensitized with T.refringens biotype Nichols and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

TABLE XX

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMAL SENSITIZED WITH T.REFRINGENS BIOTYPE NICHOLS

STR	CHA	N	MEAN	S.E.SQUD	S.E
6	1	24	0.228	0.001	0.031
6	2	24	0.318	0.001	0.031
6	3	24	0.368	0.001	0.031
6	4	24	0.413	0.001	0.031
6	5	24	0.480	0.001	0.031
6	6	24	0.876	0.001	0.031
6	7	24	0.699	0.001	0.031
6	8	24	0.135	0.001	0.031

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQUD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

T.refringens biotype Nichols showed the strongest induration at 24h. At 48h, T.refringens biotype Nichols had an induration of 61.5 mm^2 while T.refringens biotype refringens had an induration of 61.3 mm^2. At 72h, the induration of T.refringens biotype Nichols subsided to 29.0 mm^2 and T.refringens biotype refringens had 37.7 mm^2. The saline as control was of negative response.

3) The observations of skin challenge on day 30 intradermal challenge were described in figure 7c. The responses of animals on day 30 were stronger than that of day 20 and day 12. At 24h, all challenging antigens induced marked erythema and indurations. The readings are as follow: T.scoliodontum (47.0 mm^2), T.phagedenis biotype English Reiter (64.7 mm^2) T.phagedenis biotype Kazan 5 (63.0 mm^2), T.phagedenis biotype Kazan 4 (65.8 mm^2), T.denticola (79.5 mm^2), T.refringens biotype Nichols (141.0 mm^2), T.refringens biotype refringens (103.6 mm^2), and saline (55.0 mm^2). At 48h, only T.refringens biotype Nichols (60.6 mm^2) and T.refringens biotype refringens (54.7 mm^2) still provoked an induration over 40 mm^2, and all the other indurations were of no significance and the indurations were less than 40 mm^2. T.refringens biotype Nichols, the homologous antigen, still is the most potent challenging antigen on day 30 skin challenge. T.refringens biotype refringens is the second potent challenging antigen.

4) The results of all the skin reactions were pooled and summarized in Table XX. The average intensity of erythema and in indurations induced by different antigens are listed in the decreasing order: T. refringens biotype

Nichols (87.6 mm^2), T. refringens biotype refringens (69.9 mm^2), T. denticola (48.0 mm^2), T. phagedenis biotype Kazan 4 (41.3 mm^2), T. phagedenis biotype Kazan 5 (36.8 mm^2), T. phagedenis biotype English Reiter (31.8 mm^2), T. scoliodontum (32.8 mm^2) , and saline (13.5 mm^2).

(VII) T. refringens

Animals were sensitized with T. refringens biotype refringens and intradermally challenged on day 12, 20, and 30 after sensitization.

1) The skin reaction on day 12 skin challenge were shown in figure 8a. T. refringens biotype Nichols and T. refringens biotype refringens gave rise to marked erythema and induration after 24h of skin challenge. The other antigens did not induce significant indurations at 24h (greater than 40 mm^2). At 48h, only T. refringens biotype Nichols and T. refringens biotype refringens still evoked significant indurations. At 72h, all the indurations were negative. The induration of T. refringens biotype Nichols was 55.0 mm^2 at 24h ; and 39.0 mm^2 at 48h, while the induration of T. refringens biotype refringens induced an area of 83.0 mm^2 at 24h and 51.0 mm^2 at 48h. Saline as the control induced an induration of 10.0 mm^2 at 24h and subsided to zero at 48h.

2) Figure 8b showed the skin indurations on day 20 intradermal challenge. The indurations were mild and of no significance at 24h. Only T. refringens biotype Nichols and T. refringens biotype refringens induced indurations of 40 mm^2 at 24h. All the indurations subsided at 48h and 72h. Saline as the control induced an induration of 8 mm^2 at 24h and faded away at 48h.

3) Only the homologous antigen T. refringens biotype refringens showed a

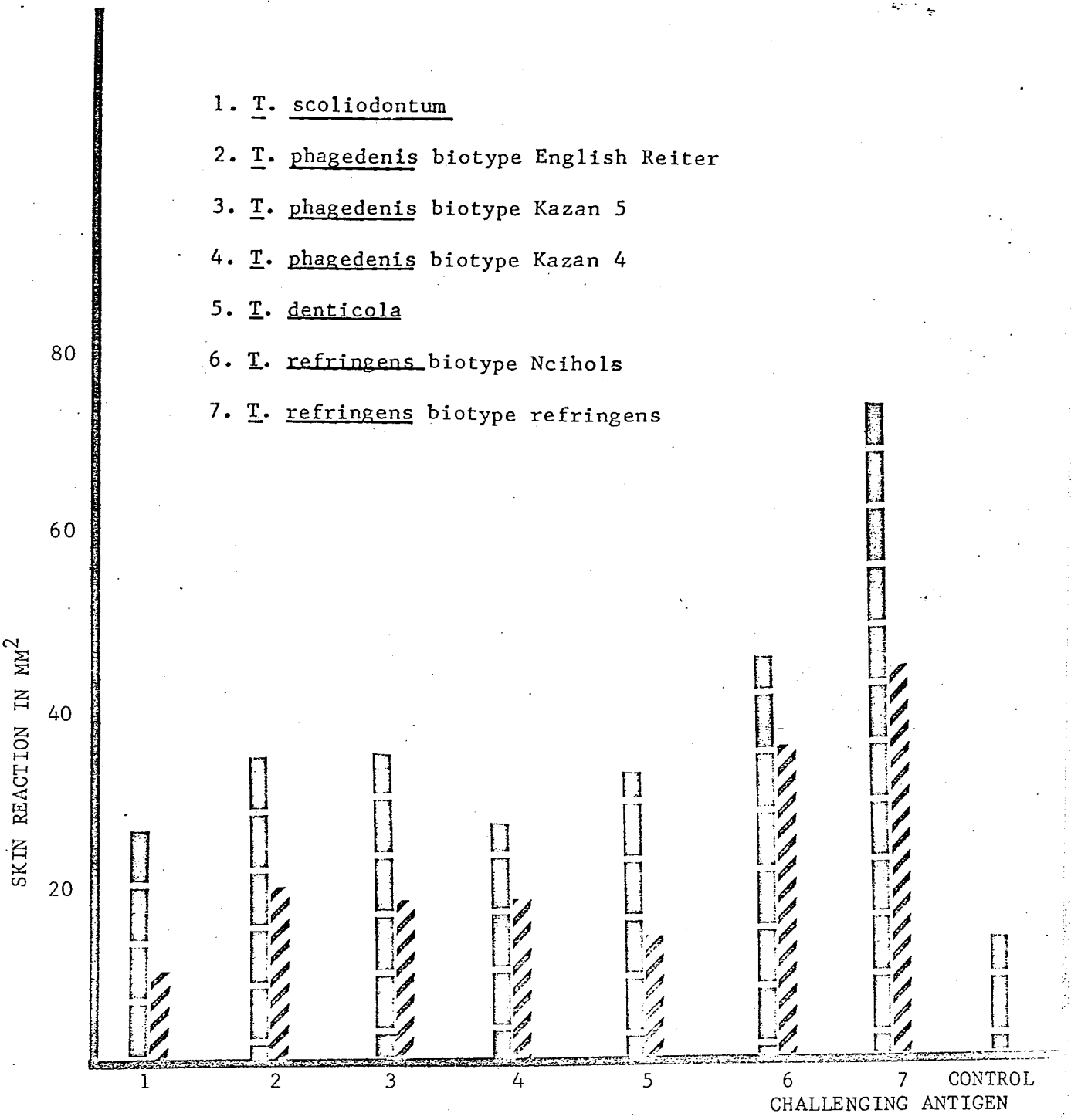


Fig.8a Skin reaction in mm^2 of guinea pigs sensitized with T. refringens biotype refringens and skin-tested on 12 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.

measurements at 24h measurements at 48h measurements at 72h

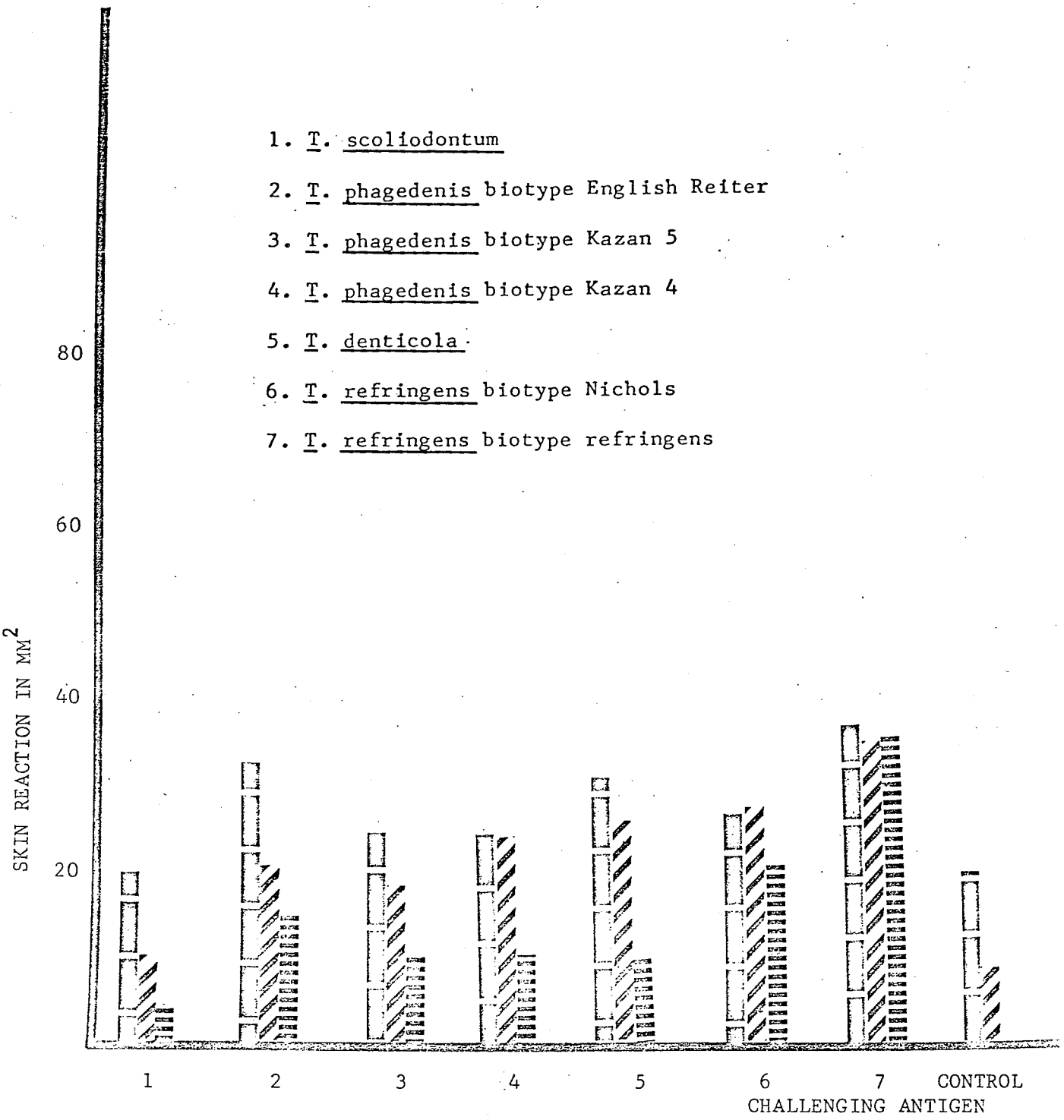


Fig.8b Skin reaction in mm^2 of guinea pigs sensitized with T. refringens biotype refringens and skin-tested on 20 days with challenging antigens. Each vertical bar represents average values for four sensitized guinea pigs.

measurements at 24h measruement at 48h measurements at 72h

marked erythem and induration on day 30 skin challenge. The results of skin reactions on day 30 have been described in figure 8c. It is clearly shown that only the homologous antigen, T. refringens biotype refringens showed a marked erythema and induration. T. refringens biotype Nichols showed only slight decrease in erythema and induration from 24h (43.0 mm^2) to 48h (41.0 mm^2) and 72h (41.0 mm^2). T. refringens biotype refringens induced an induration of 30.0 mm^2 at 24h and 32.0 mm^2 at 48h and T. phagedenis biotype Kazan 4 produced an induration of 27.0 mm^2 at 24h and 27.0 mm^2 at 48h. Both strains exhibited only slight change in the induration in the time interval observed. The other antigens did not demonstrate significant induration (less than 40 mm^2) nor gradual deminishing of skin responses.

4) The results of all skin reactions were pooled and expressed as the average of induration induced by each antigen. The averages are listed in Table XXI as follows: T. scoliodontum (17.5 mm^2), T. phagedenis biotype English Reiter (26.5 mm^2), T. phagedenis biotype Kazan 5 (26.3 mm^2), T. phagedenis biotype Kazan 4 (25.5 mm^2), T. denticola (26.2 mm^2), T. refringens biotype Nichols (38.4 mm^2), T. refringens biotype refringens (49.9 mm^2), and saline (6.6 mm^2).

Histology of dermal hypersensitivity assay.

An early acute inflammatory response with edema and polymorph infiltration was shown 24h after skin tests were performed on sensitized animals. Figure 9a and 9b showed these findings in reactions between the control guinea pig skin and the sensitized animals. Fourty-eight hours after the intradermal challenge, the test sites of heterologous

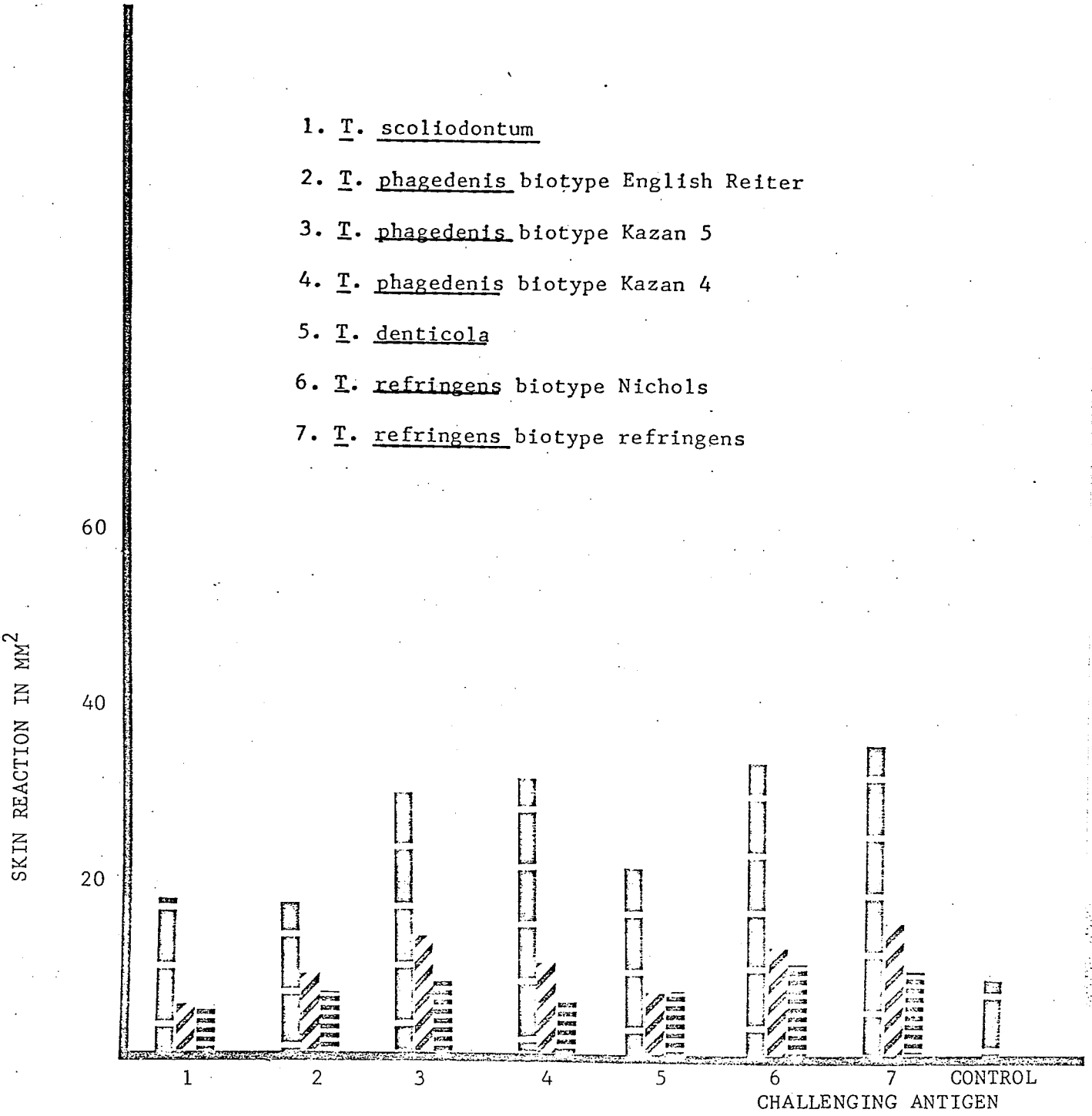


Fig.8c Skin reaction in mm^2 of guinea pigs sensitized with T.refringens biotype refringens and skin-tested on 30 days with challenging antigens. Each vertical bar represents average values for four guinea pigs.

measurement at 24h measurements at 48h measurements at 72h

TABLE XXI

THE COMPARISON OF ANTIGENS USED FOR INTRADERMAL CHALLENGE ON ANIMALS SENSITIZED WITH T.REFRINGENS BIOTYPE REFRINGENS

STR	CHA	N	MEAN	S.E.SQUD	S.E.
7	1	24	0.175	0.001	0.031
7	2	24	0.265	0.001	0.031
7	3	24	0.263	0.001	0.031
7	4	24	0.255	0.001	0.031
7	5	24	0.262	0.001	0.031
7	6	24	0.384	0.001	0.031
7	7	24	0.499	0.001	0.031
7	8	24	0.066	0.001	0.031

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQUD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

antigens showed a dense inflitration consiting predominantly of macrophages and a few lymphocytes (figure 9c). In the test sites of homologous antigen, reactions of necrosis and infiltration macrophages were found 48h after the intradermal challenge (figure 9d and figure 9e). The predominant reaction in the dermal hypersensitivity skin test proved therefore to be mononuclear cells.

Dermal hypersensitivity skin test of Treponema pallidum.

Two rabbits were infected with T.pallidum by the injection of live T.pallidum into the testicles. Twentyone days later, the testicles started to show orchitis and intradermal challenge were performed on the back of the infected rabbits. The results are as follows:

1) Antigens of cytoplasm from partially purified T.pallidum, normal rabbit testicle extraction and saline did not show any erythema and induration 24h after intradermal challenge. On the contrary, the non-pathogenic treponema antigens provoked erythema and induration. The averages of indurations induced by each challenging antigen were calculated from four skin measurements and the results are the following: T.scoliodontum (74.0 mm^2), T.phagedenis biotype English Reiter (143.0 mm^2), T.phagedenis biotype Kazan 5 (123.0 mm^2 T.phagedenis biotype Kazan 4 (129.0 mm^2), T.denticola (85.0 mm^2), T.refringens biotype Nichols (159.0 mm^2), and T.refringens biotype refringens (180.0 mm^2).

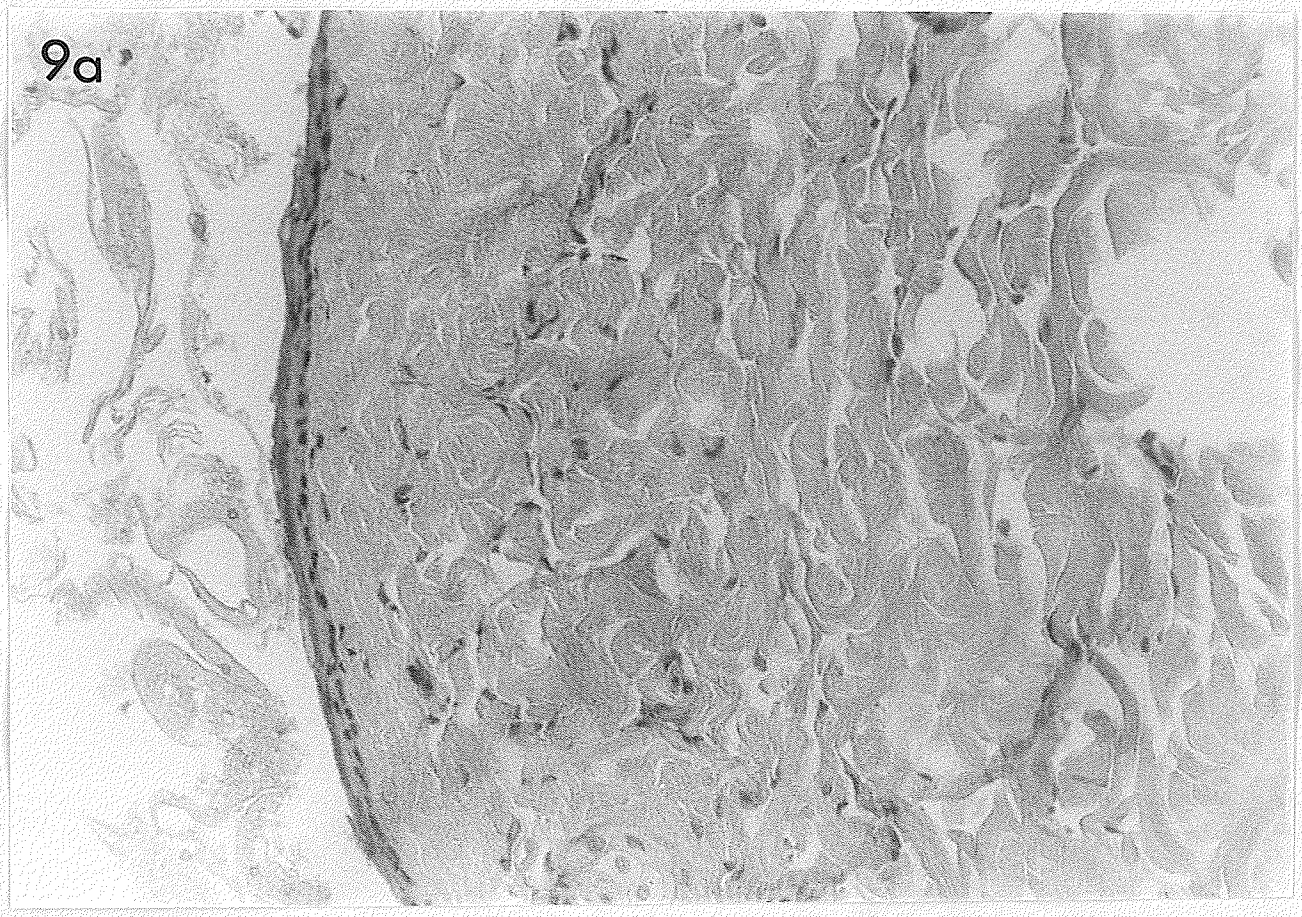
2) Fourty-eight hours afer the intradermal challenge, the challenging antigens of cytoplasm from partially purified T.pallidum, normal rabbit testicle extraction and saline still evoked no skin response while the other treponemal antigens had the indurations as follows: T.scoliodontum (53.0 mm^2), T.phagedenis biotype English Reiter (65.0 mm^2), T.phagedenis

Figure 9a

Section of dermis of control guinea pigs 24 hours after skin testing with cytoplasm of T.refringens biotype Nichols. X 640.

Figure 9b

Section of dermis of guinea pigs sensitized with T.refringens biotype Nichols 24 hours after skin testing on post-sensitization day 30. Skin tested with cytoplasm of T.refringens biotype Nichols. Tissue shows acute inflammatory response with edema and polymorph infiltration. X 640.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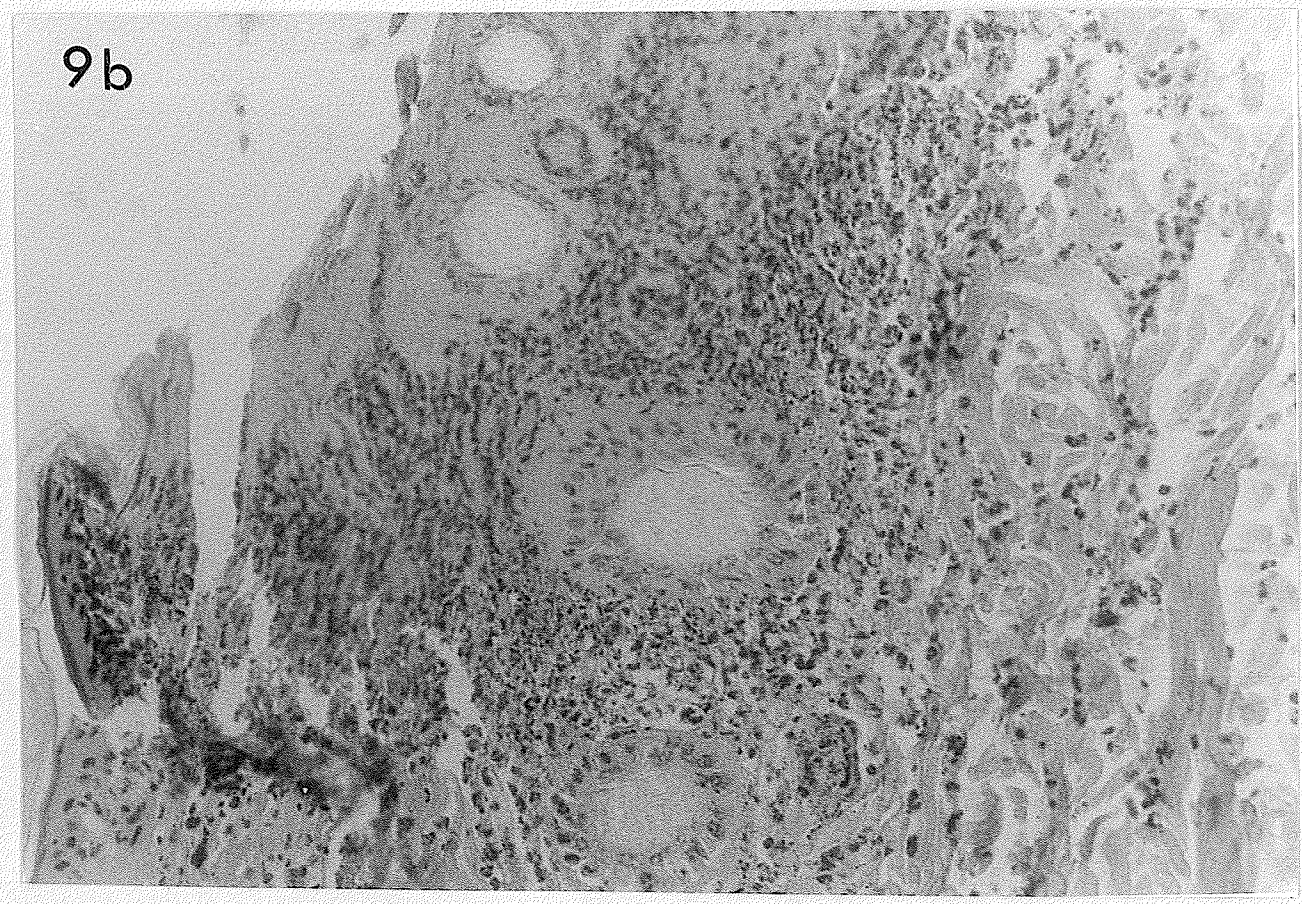
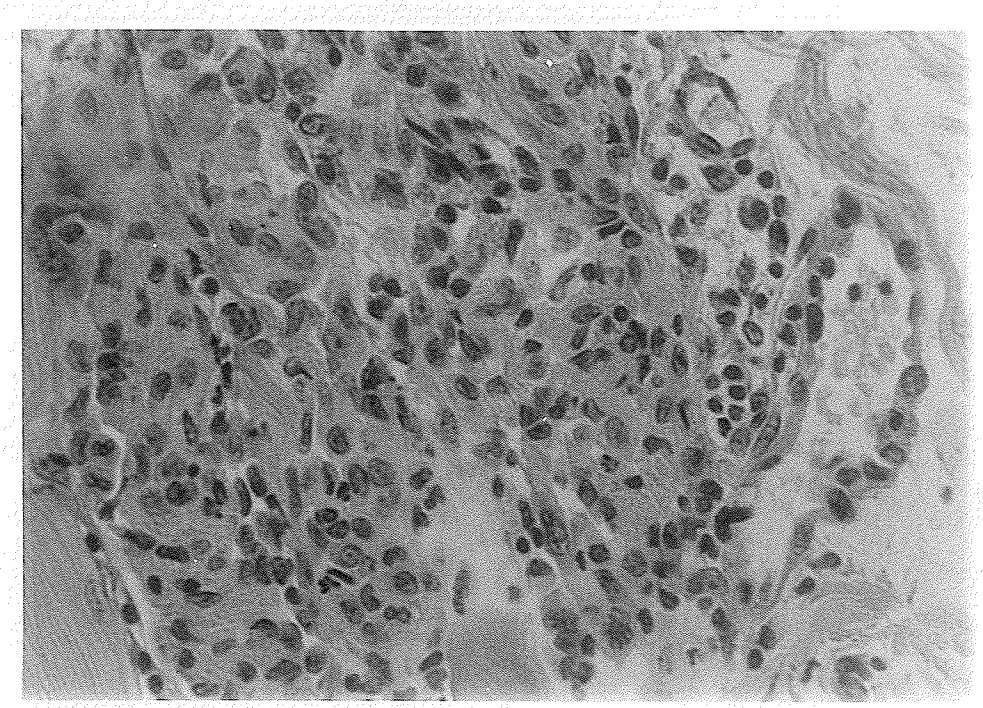

9b

Figure 9c

Section of dermis from guinea pig sensitized with T.refringens biotype Nichols 48 hours after skin testing on post-sensitization day 30. Skin tested with T.refringens biotype refringens. Tissue shows dense infiltration with macrophage. X 1300.

Figure 9d

Section of dermis from guinea pig sensitized with T.phagedenis biotype English Reiter 48 hours after skin testing on post-sensitization day 30. Skin tested with the homologous antigen T.phagedenis biotype English Reiter. Tissue shows edema of epidermis and infiltration of macrophage. X 640.



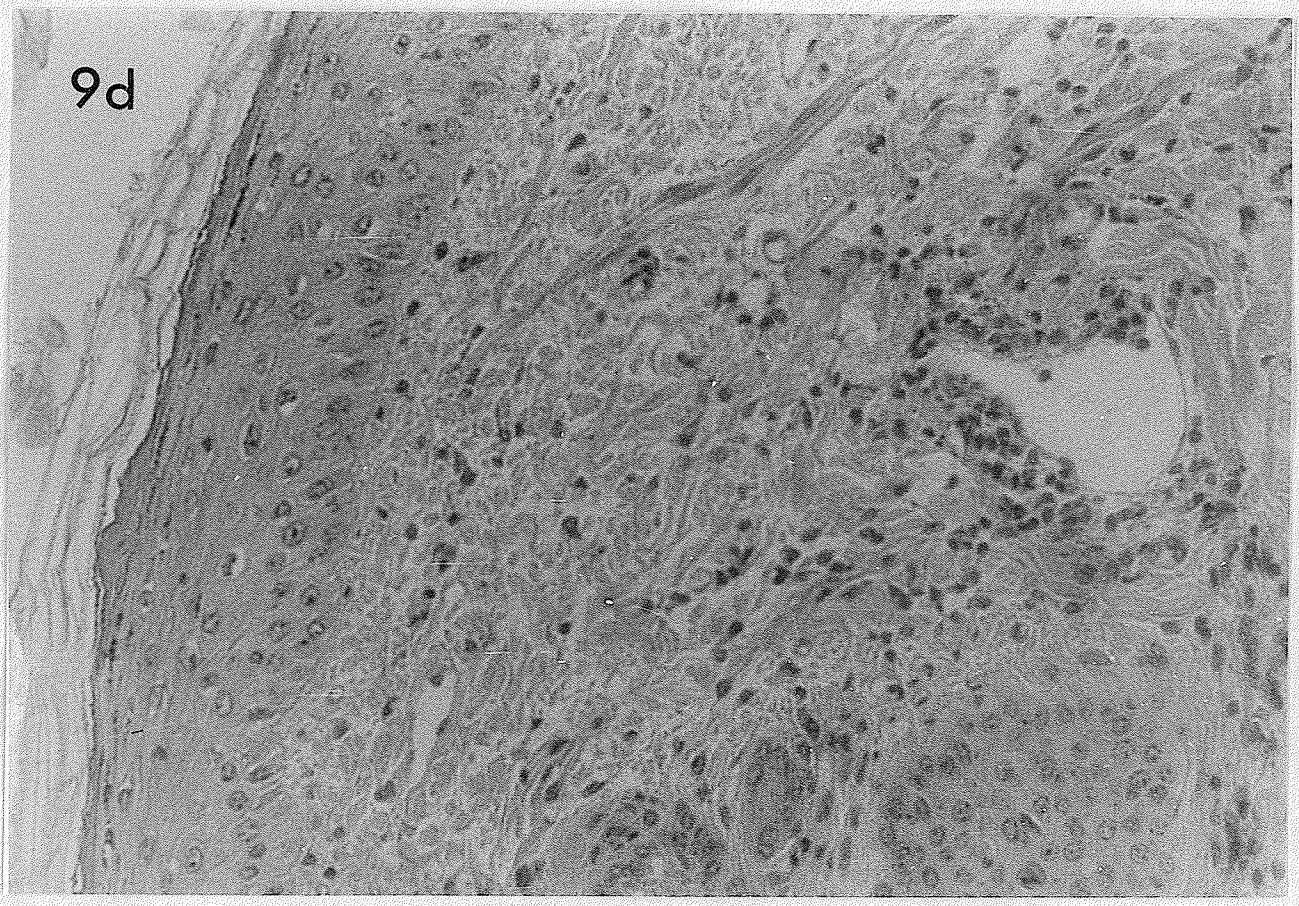
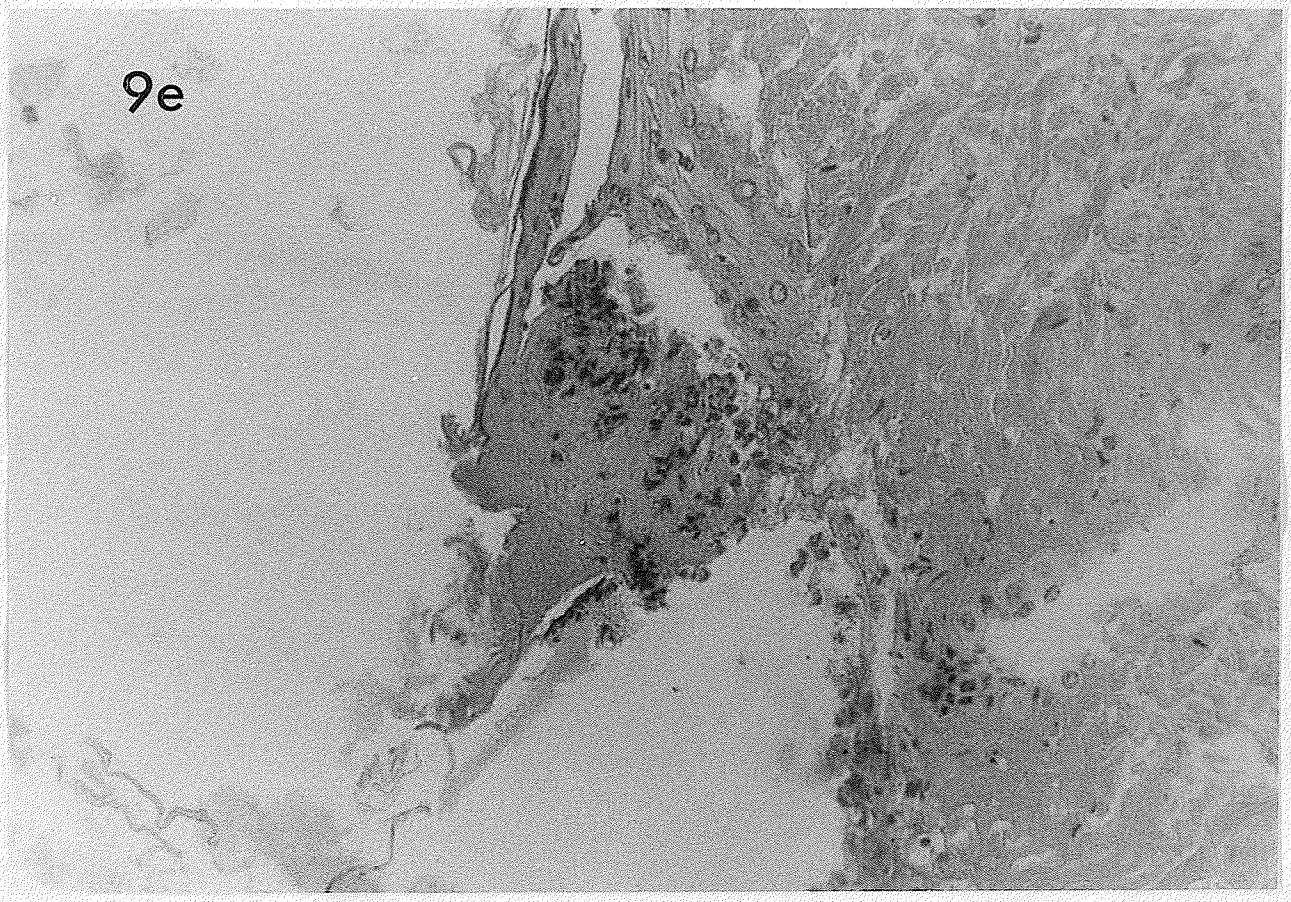

9d

Figure 9e

Section of dermis from guinea pig sensitized with T.phagedenis biotype English Reiter 48 hours after skin testing on post-sensitization day 30. Skin tested with the homologous antigen T.phagedenis biotype English Reiter. Tissue shows the reaction of necrosis. X 640.


9e

biotype Kazan 5 (49.0 mm^2), T.phagedenis biotype Kazan 4 (146.0 mm^2), T.denticola (31.0 mm^2), T.refringens biotype Nichols (100.0 mm^2), and T.refringens biotype refringens (100.0 mm^2).

3)Seventy-two hours after the intradermal challenge, all the indurations subsided to nil.

The mutual relationships among treponemal antigens in skin test.

The skin reactions on the 12th day intradermal challenge were usually mild. Therefore the results obtained form the 20th and 30th day intradermal challenges were used in the calculation regarding the mutual relationships among treponemal antigens.

The Duncan's New Multiple-Range test was used for finding the significant relationships among treponemal antigens. A significant student's test range of 5% was selected. Each antigen was in turn assumed to be one hundred percent and a simple percentage calculation was used for computing the comparative percentage of each treponemal antigen. Seventy percent was selected as the minimal percentage to show the positive relationship. The results are separatly summarized according to the date of challenge after sensitization, and are shown as below:

(1) The 20th day intradermal challenge

The averages of indurations induced by different treponemal antigens on the 20th day skin test were: T.scoliodontum (18.5 mm^2), T.phagedenis biotype English Reiter (41.8 mm^2), T.phagedenis biotype Kazan 5 (20.5 mm^2), T.phagedenis biotype Kazan 4 (33.2 mm^2), T. denticola (38.1 mm^2), T.refringens biotype Nichols (49.9 mm^2), and T.refringens biotype refringens (19.9 mm^2). These averages of induration were used for the Duncan's New multiple-Range test and comparative per-

centage calculation. The results showed that there was no significant differences (α= 0.05) between T.phagedenis biotype Kazan 5 and T.scoliodontu (comparative percentage 90 and 90, see Table XXII); T.phagedenis biotype Kazan 4 and T.phagedenis biotype English Reiter (comparative percentage 75 and 79); T.denticola and T.phagedenis biotype English Reiter (comparativ percentage 91 and 91); T.denticola and T.phagedenis biotype Kazan 4 (comparative percentage 86 and 87); T.refringens biotype Nichols and T.phagedenis biotype English Reiter (comparative percentage 81 and 83); T.refringens biotype Nichols and T.denticola (comparative 70 and 76); T.refringens biotype refringens and T.scoliodontum (comparative percentage 92 and 93); and T.refringens and T.phagedenis biotype Kazan 5 (comparative percentage 97 and 97). The other treponemal antigens did not show any mutual relationships according to the Duncan's New Multiple-Range test and their corresponding comparative percentage were less than 70%.

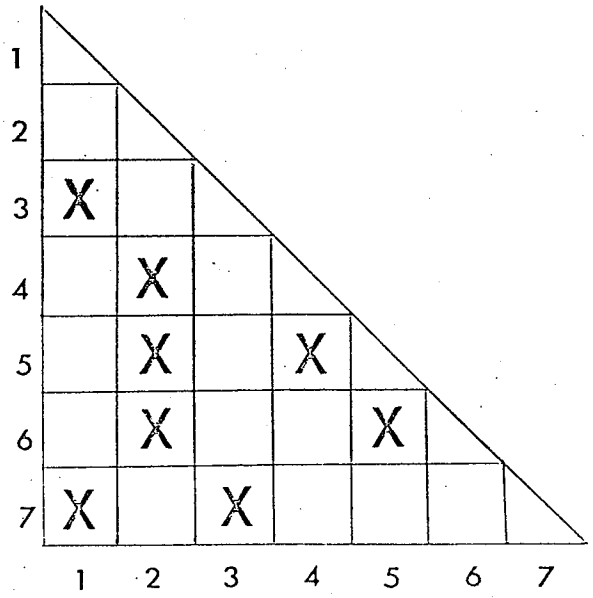
(2) The 30th day intradermal challenge

The average of induration induced by different treponemal antigens in the 30th day skin test were T.scoliodontum (38.2 mm^2), T.phagedenis biotype English Reiter (33.9 mm^2), T.phagedenis biotype Kazan 5 (16.1 mm^2 T.phagedenis biotype Kazan 4 (26.8 mm^2), T.denticola (32.7 mm^2), T.refringens biotype Nichols (57.3 mm^2), and T.refringens biotype refringens (26.8 mm^2). From the Duncan's New Multiple-Range test and comparative percentage calculation, it showed that there was no significant difference (α= 0.05) between T.phagedenis biotype English Reiter and T.scoliodontum (comparative percentage 88 and 99, see Table XXIII); T.phagedenis biotype Kazan 4 and T.scoliodontum (comparative percentage 70 and 58); T.phagedenis biotype Kazan 4 and T.phagedenis biotype English Reiter (comparative percentage 79 amd 74); T.phagedenis biotype Kazan 4 and T.phagedenis biotype

Kazan 5 (comparative percentage 34 and 60); T.denticola and T.scoliodontum (comparative percentage 85 and 84), T.denticola and T.phagedenis biotype English Reiter (comparative percentage 96 and 97); T.denticola and T. phagedenis biotype Kazan 4 (comparative percentage 78 and 81); T.refringens biotype refringens and T.scoliodontum (comparative percentage 70 and 58); T.refringens biotype refringens andT.phagedenis biotype English Reiter (comparative percentage 79 and 74); T.refringens biotype refringens and T.phagedenis biotype Kazan 5 (comparative percentage 34 and 60); T.refringens biotype refringens and T.phagedenis biotype Kazan 4 (comparative percentage 100 and 100); and T.refringens biotype refringens and T.denticola (comparative percentage 81 and 78). There was no mutual relationships among the other treponemal antigens and their corresponding comparative percentage were less than 70%.

TABLE XXII

THE DUNCAN'S NEW MULTIPLE-RANGE TEST AND COMPARATIVE PERCENTAGE OF THE SKIN REACTIONS OF THE 20TH DAY INTRADERMAL CHALLENGE



a. The Duncan's New Multiple-Range test.

X: There is no significant difference from each other ($\alpha = 0.05$).

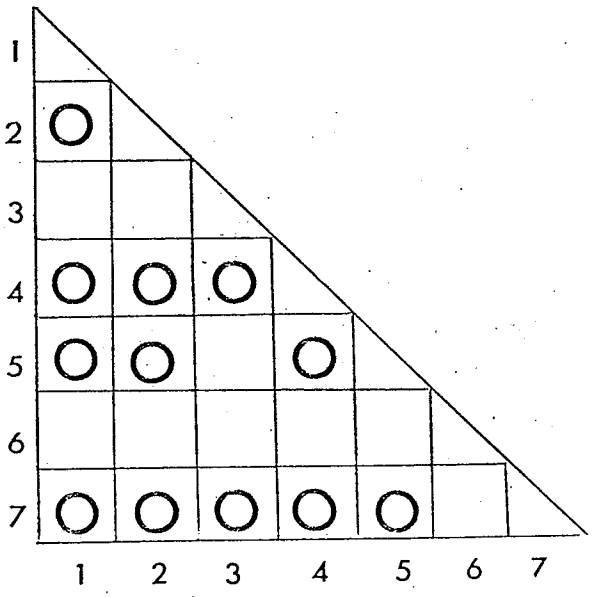
1	100	44	90	55	48	37	92
2	25	100	3	75	91	83	10
3	90	49	100	61	53	41	97
4	21	79	39	100	87	66	34
5	5	91	15	86	100	76	9
6	31	81	43	50	70	100	50
7	93	47	97	57	52	39	100
	1	2	3	4	5	6	7

b. The comparative percentage of treponemal antigens, percentage higher than 70 were positive for mutual relationship.

1. T. scoliodontum
2. T. phagedenis biotype English Reiter
3. T. phagedenis biotype Kazan 5
4. T. phagedenis biotype Kazan 4
5. T. denticola
6. T. refringens biotype Nichols
7. T. refringens biotype refringens

TABLE XXIII

THE DUNCAN'S NEW MULTIPLE-RANGE TEST AND COMPARATIVE PERCENTAGE OF THE SKIN REACTIONS OF THE 30 DAYS INTRADERMAL CHALLENGE



a. The Duncan's New Multiple-Range test.

○: There is no significant difference from each other ($\alpha = 0.05$).

1	100	88	37	58	84	66	58
2	88	100	10	74	97	59	74
3	42	47	100	60	49	28	60
4	70	79	34	100	81	46	100
5	85	96	3	78	100	57	78
6	50	31	55	13	25	100	13
7	70	79	34	100	81	46	100
	1	2	3	4	5	6	7

b. The comparative percentage of treponemal antigens; percentage higher than 70 were postive for mutual relationship.

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens

II. Macrophage migration inhibition test

The computer analysis of variance on the data obtained from the macrophage migration inhibition testing are summarized in Table XXIV. The following observation are made:

Macrophage migration inhibition tests carried out on the animals immunized with the cytoplasm of different treponemes showed statistically significant difference in the extent of macrophage migration inhibition (MMI). Different strains gave rise to different degrees of sensitization indicated by MMI tests ($F = 30.263$).

The sensitized animal showed different extent of MMI which varied significantly ($\alpha = 0.05$) depending on the period of sensitization (namely 12, 20, and 30 days) and on the strains used for immunization. Relations between individual strains and the period of sensitization for MMI test are different ($F = 45.942$).

The antigens used for the MMI test produced significant ($\alpha = 0.05$) different macrophage migration inhibition ($F = 21.967$).

Animals sensitized with strains gave rise to significant ($\alpha = 0.05$) different response to different antigens used for MMI test. The relations between each strain used for sensitization and antigens used for MMI test were different ($F = 8.409$).

Comparison of the degree of sensitization among strains for macrophage migration inhibition test

Table XXV shows mean and standard errors for the macrophage migration inhibition tests. The mean of the area of macrophage migration of each strain was computed from 96 reading (four replications form one

TABLE XXIV

ANALYSIS OF VARIANCE OF THE NUMERICAL DATA OBTAINED FROM MACROPHAGE MIGRATION INHIBITION ASSAYS

SOURCE OF VARIATION	DF	SS	MS	F
STR	6	736.9377	122.8230	30.263
STR DAY	12	2237.4365	186.4530	45.942
CHA	7	112.7306	16.1044	21.967
STR CHA	42	258.9226	6.1648	8.407

STR: strain for sensitization

CHA: challenging antigens

DF: degree of freedom

SS: sum of square

MS: mean of square

F: F ratio

TABLE XXV

MEANS AND STANDARD ERRORS OF THE DATA FROM MACROPHAGE MIGRATION INHIBITION TO ALL TREPONEMAL CYTOPLASMS IN GUINEA PIGS SENSITIZED WITH THE CYTOPLASM OF INDIVIDUAL STRAINS

	N	MEAN	S.E.SQRD	S.E.
STR				
1	96	2.504	0.042	0.206
2	96	3.866	0.042	0.206
3	96	3.243	0.042	0.206
4	96	5.948	0.042	0.206
5	96	3.076	0.042	0.206
6	96	4.157	0.042	0.206
7	96	3.105	0.042	0.206

STR: cytoplasm of all sensitizing strains
1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

related time, 24h, in three different days, 12, 20, and 30 days, respectively (4 x 3), seven antigens plus control (8)). It was found that T. scoliodontum induced the smallest area of macrophage migration (2.504 in^2) which is equivalent to the greatest inhibition of macrophage migration in this series of experiment. The average of macrophage migrations of other strains are arranged in increasing order (i.e., decreasing order in migration inhibition) as follows: T. denticola (3.076 in^2), T. refringens biotype refringens (3.105 in^2), T. phagedenis biotype Kazan 5 (3,243 in^2), T. phagedenis biotype English Reiter (3.866 in^2), T. refringens biotype Nichols (4.157 in^2), and T. phagedenis ciotype Kazan 4 (5.948 in^2).

Comparison of the degree of macrophage migration inhibition by different antigens used for the test

Table XXVI summarized the means and standard errors of the areas of migration of macrophage of the sensitized animals when different antigens were used for the test. The mean of the area of macrophage migration of each antigen was calculated from 84 readings (four replications from one related time, 24h, in three different days, 12, 20, and 30, respectively (4 x 3), seven antigens (7)). It can be seen that T. phagedenis biotype Kazan 4 gave the smallest area of macrophage migration by producing an average area of 3.134 in^2 and it is the highest degree of macrophage migration inhibition. The macrophage migration area in the presence of other antigens are as follows: T. scoliodontum (3.594 in^2), T. phagedenis biotype English Reiter (3.593 in^2), T. phagedenis biotype Kazan 5 (3.916 in^2), T. denticola (3.573 in^2), T. refringens biotype Nichols (3.688 in^2), and T. refringens biotype refringens (3.464 in^2).

TABLE XXVI

MEAN AND STANDARD ERRORS OF THE DATA OBTAINED FROM MACROPHAGE MIGRATION INHIBITION TESTS TO A SINGLE CYTOPLASM IN GUINEA PIGS SENSITIZED WITH CYTOPLASMS OF DIFFERENT TREPONEMES

	N	MEAN	S.E.SQRD	S.E.
CHA				
1	84	3.594	0.009	0.093
2	84	3.593	0.009	0.093
3	84	3.916	0.009	0.093
4	84	3.134	0.009	0.093
5	84	3.573	0.009	0.093
6	84	3.688	0.009	0.093
7	84	3.464	0.009	0.093
8	84	4.637	0.009	0.093

CHA: challenging antigens
1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

The saline was used for control and gave an area of 4.637 in^2 of migration.

Macrophage migration inhibition (MMI) by cytoplasms used for the test

Animals were immunized and macrophage migration inhibition tests were carried out after the 12th, 20th, and 30th days of intradermal challenge The areas of migration of macrophage were measured and the macrophage migratic inhibition indices were calculated. Figures 10 and 11 showed the positive and negative migration of macrophages. The findings of the tests of the individual strain are the following:

(I) T.scoliodontum

Animals were sensitized with T.scoliodontum and macrophage migration inhibition tests were carried out after the 12th, 20th, and 30th day of intradermal challenge. It can be seen from figure 12 that the macrophage migration inhibtion indices of T.scoliodontum started from 19% (after the 12th day skin test) to 36% (after the 20th day skin test) and 33% (after the 30th day skin test). T.phagedenis biotype English Reiter showed a significnat inhibition of migration of macrophage (macrophage migration inhibition index 30%) after the 30th day skin test. T.phagedenis biotype Kazan 4 with an index of 5% after the 12th day skin test and increased to 22% (after the 20th day skin test) and 39% (after the 30th day skin test). T.denticola only showed a significant inhibition after the 20th day skin test by having an index of 24%. T.refringens biotype refringens had the highest index after the 12th day and 30th day skin test, 44% and 41%, respectively The index of T.refringens biotype refringens after the 20th day skin test was 19%. The indices of the antigens were of no significance (less than 20%).

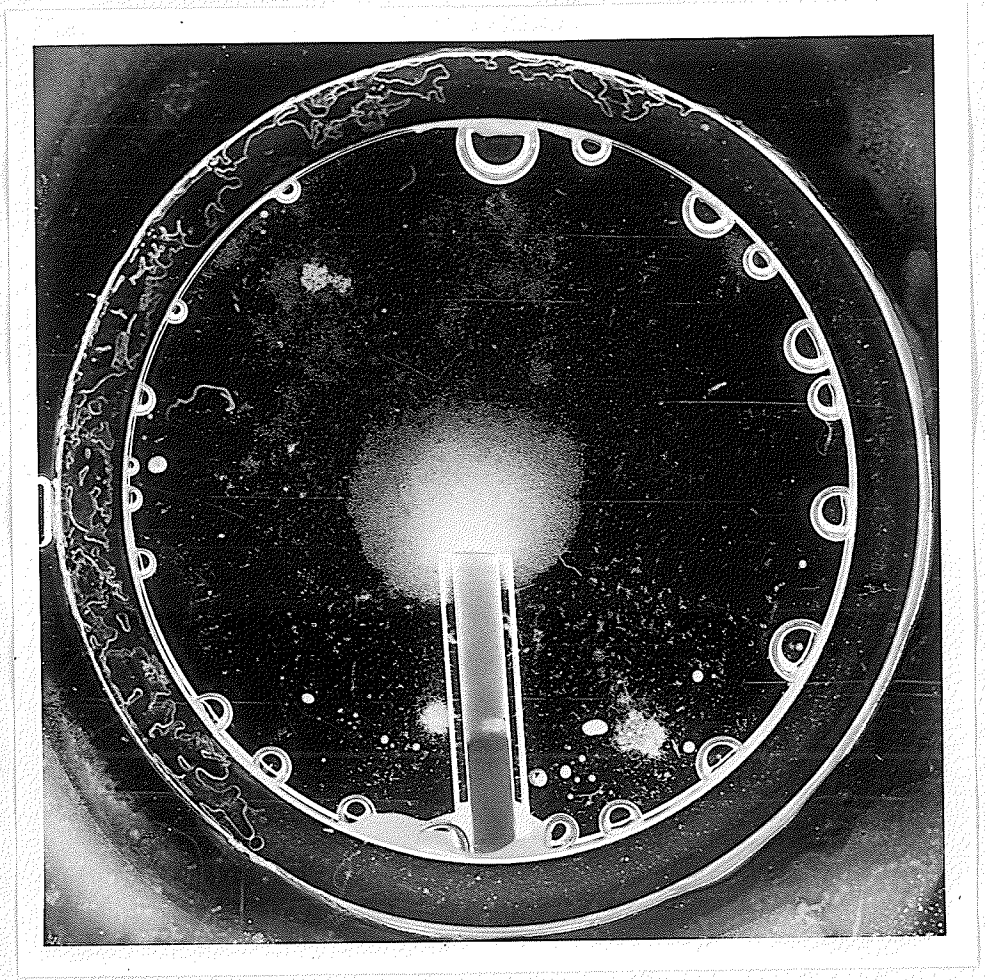


Fig.10 Uninhibited macrophage migration showed in the macrophage migration inhibition test performed with the treponemal cytoplasms.

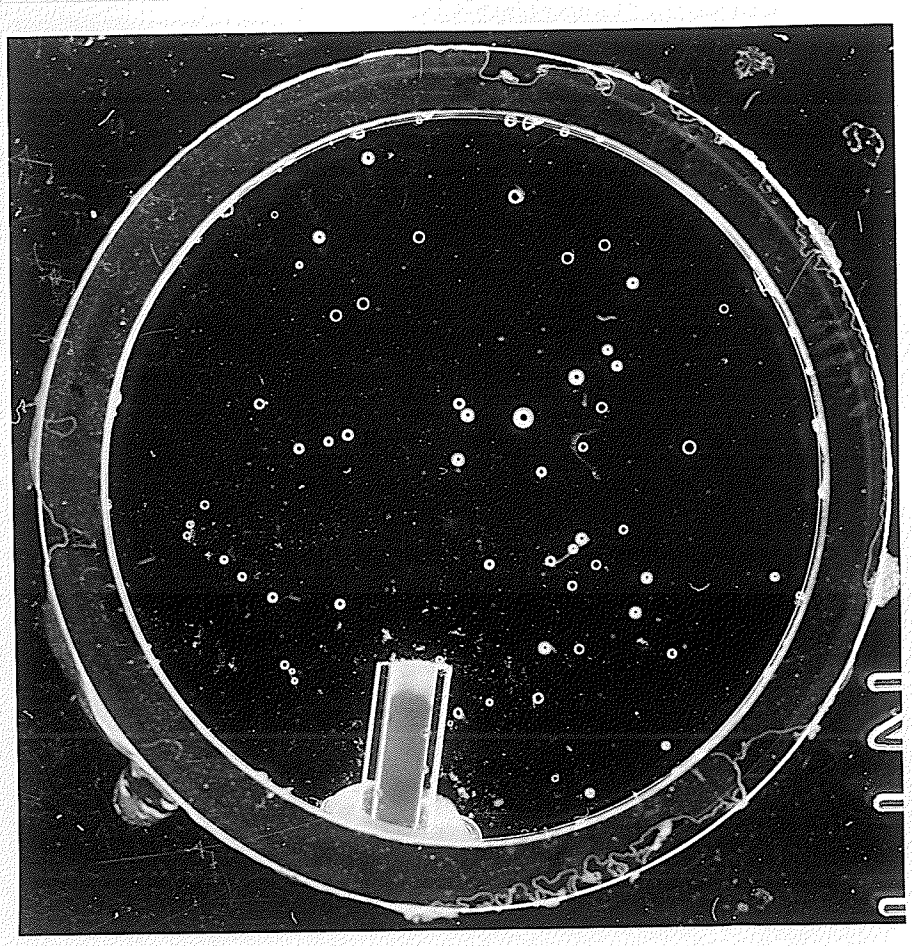


Fig.11 Inhibited macrophage migration showed in the macrophage migration inhibition test performed with the treponemal cytoplasms.

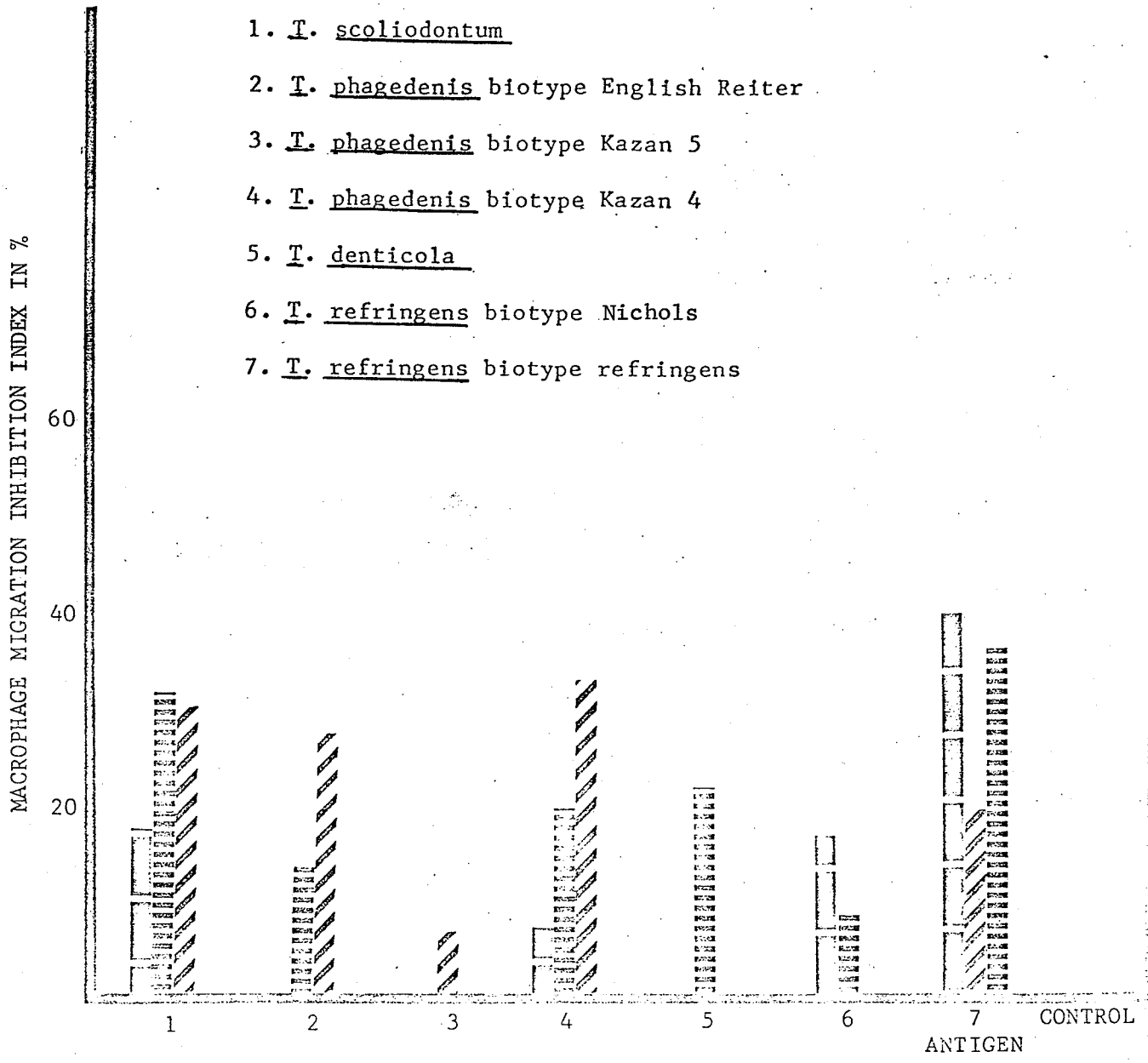


Fig.12 Macrophage migration inhibition indices of animals sensitized with T.scoliodontum.

after the 12th day intradermal challenge

after the 20th day intradermal challenge

after the 30th day intradermal challenge

The comparisons of the area of migration of macrophage from animals sensitized with T.scoliodontum in the presence of different antigens were listed in Table XXVII in an increasing order (i.e., decreasing order in migration inhibition) as follows: 1.865 in^2 (T.scoliodontum), 2.212 in^2 (T.phagedenis biotype Kazan 4), 2.438 in^2 (T.phagedenis biotype English Reiter), 2.718 in^2 (T.denticola), 2.817 in^2 (T.phagedenis biotype Kazan 5), and 3.162 in^2 (without antigen).

(II) T.phagedenis biotype English Reiter

Animals sensitized with T.phagedenis biotype English Reiter and macrophage migration inhibition (MMI) tests were carried out after the 12th, 20th, and 30th day skin tests. The MMI indices of different antigens were shown in figure 13. T.refringens biotype Nichols and T.scoliodontum showed significant inhibition of migration of macrophage (MMI index greater than 20%) after the 30th day skin test. Their MMI indices were 20% (T.scoliodontum) and 36% (T.refringens biotype Nichols). However, the homologous antigen T.phagedenis biotype English Reiter had the index of 18% after the 12th day skin test, 20% after the 20th day skin test, and 50% after the 30th day skin test. T.phagedenis biotype Kazan 5 gave rise to an index of 24% after the 12th day skin test and failed to inhibit the migration of macrophage after the 20th and 30th day skin tests. T.phagedenis biotype Kazan 4 was the most potent antigen to inhibit the macrophage migration by having an MMI index of 24% after the 12th day skin tests, 29% after the 20th day skin test, and 38% after the 30th day skin test. T.denticola and T.refringens biotype refringens had the indices of 20%and 36%, respectively, after the 12th day skin test but failed to inhibit the migration of macrophage after the 20th day skin test. However, both antigens showed significant in-

TABLE XXVII

THE COMPARISONS OF THE AREA OF MIGRATION OF MACROPHAGE FROM ANIMALS SENSITIZED WITH T.SCOLIODONTUM

STR	CHA	N	MEAN	S.E.SQRD	S.E.
1	1	12	2.092	0.061	0.247
1	2	12	2.438	0.061	0.247
1	3	12	2.817	0.061	0.247
1	4	12	2.212	0.061	0.247
1	5	12	2.722	0.061	0.247
1	6	12	2.718	0.061	0.247
1	7	12	1.865	0.061	0.247
1	8	12	3.162	0.061	0.247

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype refringens
7. T.refringens biotype refringens
8. saline as the control

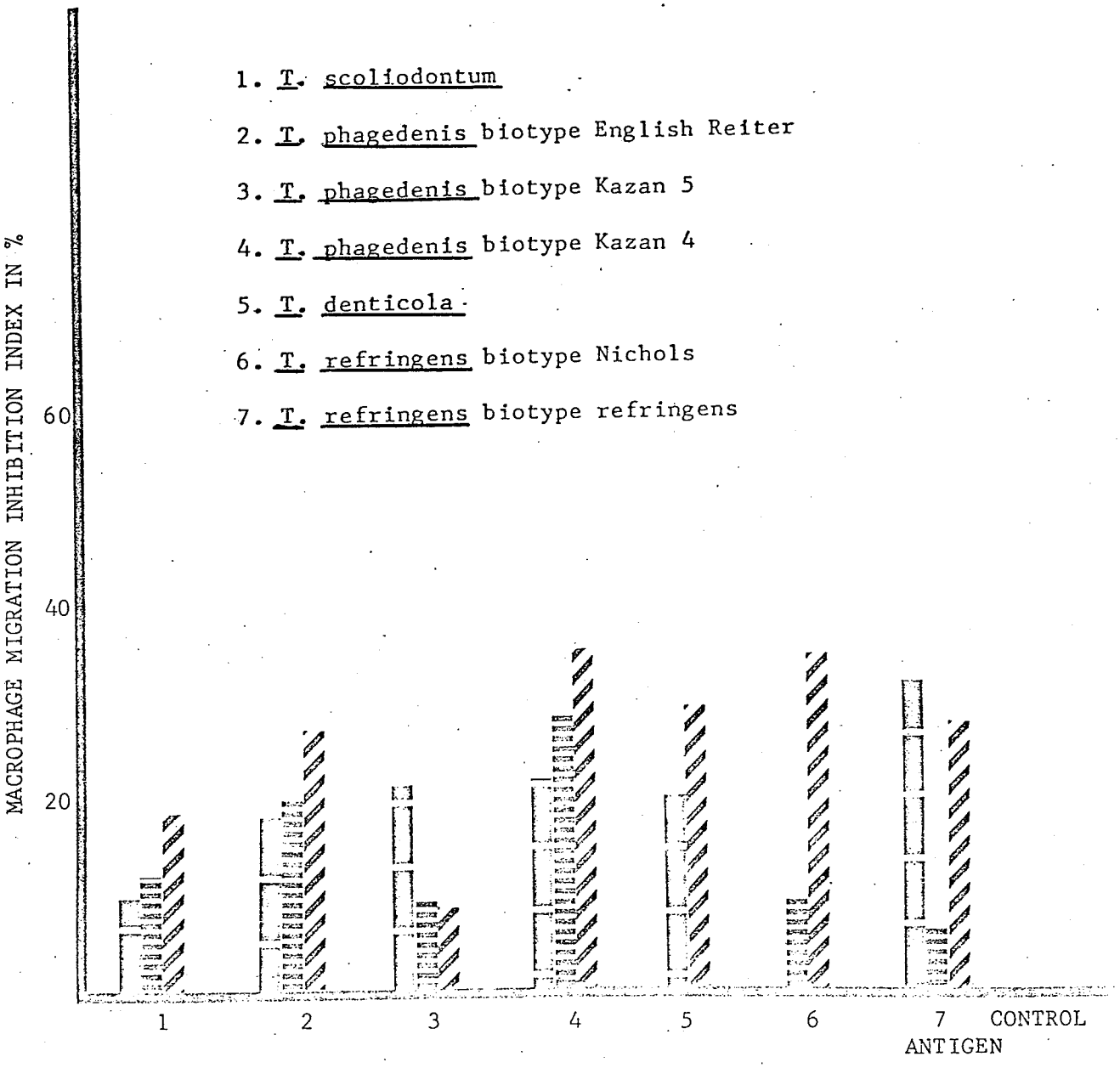


Fig.13 Macrophage migration inhibition indices of animals sensitized with T.phagedenis biotype English Reiter.

after the 12th day intradermal challenge

after the 20th day intradermal challenge

after the 30th day intradermal challenge

hibition of migration of macrophage after the 30th day skin test (28% of MMI index for T. denticola and 25% for T. refringens biotype refringens).

The average areas of macrophage migration in the presence of different antigens were listed in Table XXVIII as follows: 3.994 in^2 (T. scoliodontum, 3.057 in^2 (T. phagedenis biotype English Reiter), 4.011 in^2 (T. phagedenis biotype Kazan 5), 3,200 in^2 (T. phagedenis biotype Kazan 4), 4.501 in^2 (T. denticola), 3.727 in^2 (T. refringens biotype Nichols), 3.799 in^2 (T. refringens biotype refringens), 5.089 in^2 (without antigen).

(III) T. phagedenis biotype Kazan 5

Animals sensitized with T.phagedenis biotype Kazan 5 and MMI tests were carried out after the 12th, 20th, and 30th day intradermal challenge.

The macrophage migration inhibition indices of different antigens were showed in figure 14. All antigens failed to inhibit migration of macrophage after the 12th day intradermal challenge and their MMI indices were of no significance. Except T.phagedenis biotype English Reiter, all the other antigens showed significant inhibition of macrophage migration after the 20th day skin test. The MMI indices of these antigens were: 21% (T.scoliodontum), 31% (T.phagedenis biotype Kazan 5), 34% (T.phagedenis biotype Kazan 4), 22% (T.denticola), 43% (T.refringens biotype Nichols); and 27% (T.refringens biotype refringens). After the 30th day skin test, only T.phagedenis biotype English Reiter, T.phagedenis biotype Kazan 4, and T.refringens showed significant inhibition of the migration of macrophage and their MMI indices were 21% (T.phagedenis biotype English Reiter), 21% (T.phagedenis biotype Kazan 4), and 23% (T.refringens biotype refringens).

TABLE XXVIII

THE COMPARISONS OF THE AREA OF MIGRATION OF MACROPHAGE FROM ANIMALS SENSITIZED WITH T.PHAGEDENIS BIOTYPE ENGLISH REITER

		N	MEAN	S.E.SQRD	S.E.
STR	CHA				
2	1	12	3.994	0.061	0.247
2	2	12	3.057	0.061	0.247
2	3	12	4.011	0.061	0.247
2	4	12	3.200	0.061	0.247
2	5	12	4.051	0.061	0.247
2	6	12	3.727	0.061	0.247
2	7	12	3.799	0.061	0.247
2	8	12	5.089	0.061	0.247

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

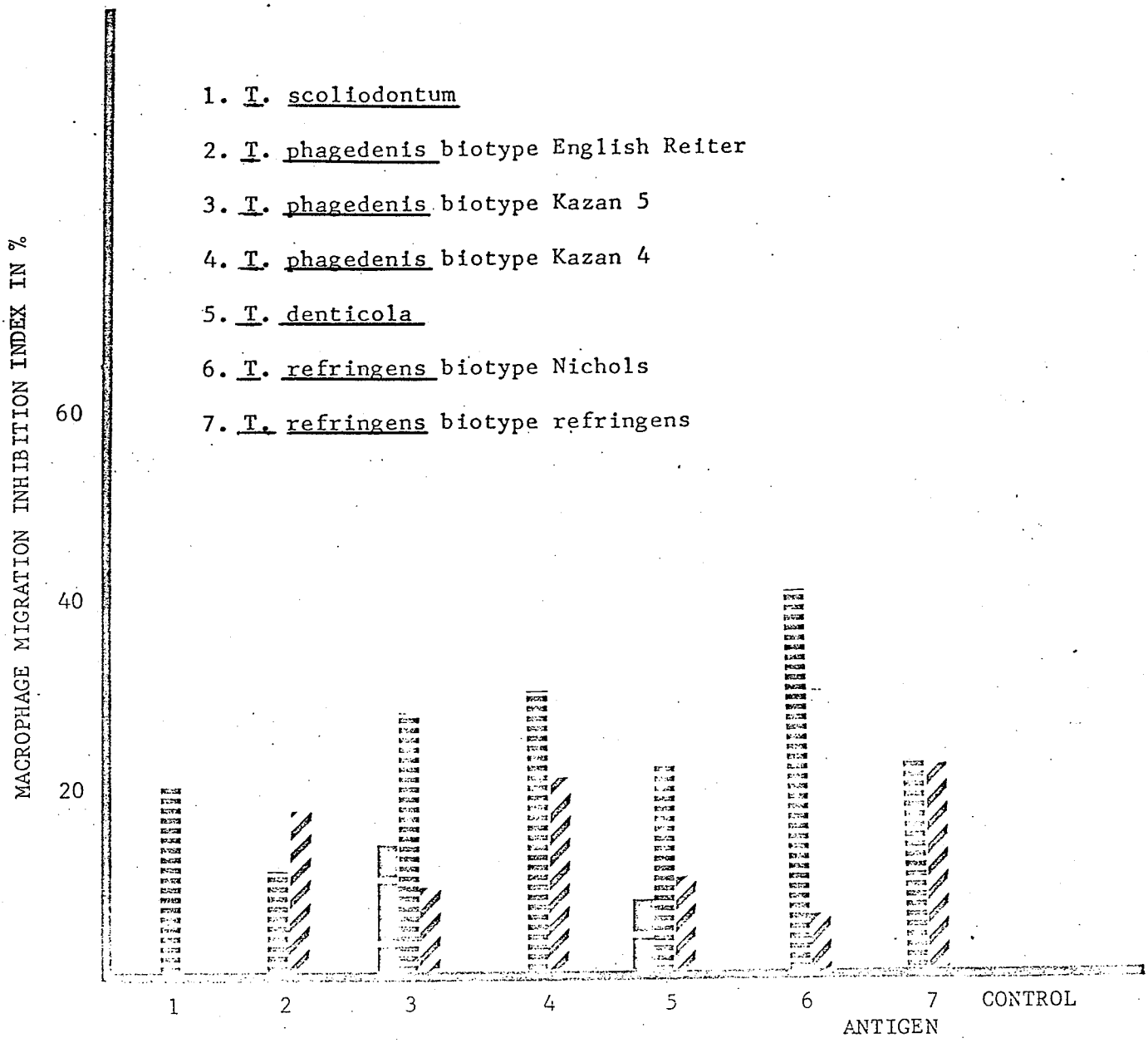


Fig.14 Macrophage migration inhibition indices of animals sensitized with T.phagedenis biotype Kazan 5.

after the 12th day intradermal challenge

after the 20th day intradermal challenge

after the 30th day intradermal challenge

The comparisons of averages area of migration of macrophage in the presence of different antigens were shown in Table XXIX. The average areas were: 3.772 in^2 (T.scoliodontum), 3.112 in^2 (T.phagedenis biotype Kazan 4), 3.294 in^2 (T.denticola), 3.090 in^2 (T.refringens biotype Nichols 3.032 in^2 (T.refringens biotype refringens), and 3.160 in^2 (without antigen T.phagedenis biotype Kazan 4 induced the smallest area of migration, that is equivalent to the highest degree of inhibition of macrophage migration, therefore, it is the most potent antigen to induce cellular reactivity.

(IV) T.phagedenis biotype Kazan 4

Animals immunized with T.phagedenis biotype Kazan 4 and MMI tests were carried out after the 12th, 20th, and 30th day intradermal challenge.

The MMI indices of different antigens used for macrophage migration inhibition tests were shown in figure 15. All antigens failed to inhibit the migration of macrophage form sensitized animals after the 12th day skin test. However, a few significant MMI indices (greater than 20%) were shown after the 20th day intradermal challenge. These significant indices were : 32% (T.phagedenis biotype English Reiter), 47% (T.phagedenis biotype Kazan 5), 57% the homologous antigen, T.phagedenis biotype Kazan 4), and 40% (T.refringens biotype refringens). The indices of other antigens were insignificant. After the 30th day intradermal challenge, most of the indices showed a great increase and they were: T.scoliodontum (52%), T.phagedenis biotype English Reiter (46%), T. phagedenis biotype Kazan 5 (47%), T. phagedenis biotype Kazan 4 (43%), T. denticola (45%), T. refringens biotype Nichols (1%), and T. refringens biotype refringens (27%).

TABLE XXIX

THE COMPARISONS OF THE AREA OF MIGRATION OF MACROPHAGE FROM ANIMAL SENSITIZED WITH T.PHAGEDENIS BIOTYPE KAZAN 5

		N	MEAN	S.E.SQRD	S.E.
STR	CHA				
3	1	12	3.772	0.061	0.247
3	2	12	3.409	0.061	0.247
3	3	12	3.112	0.061	0.247
3	4	12	3.022	0.061	0.247
3	5	12	3.297	0.061	0.247
3	6	12	3.090	0.061	0.247
3	7	12	3.082	0.061	0.247
3	8	12	3.160	0.061	0.247

STR: strain for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

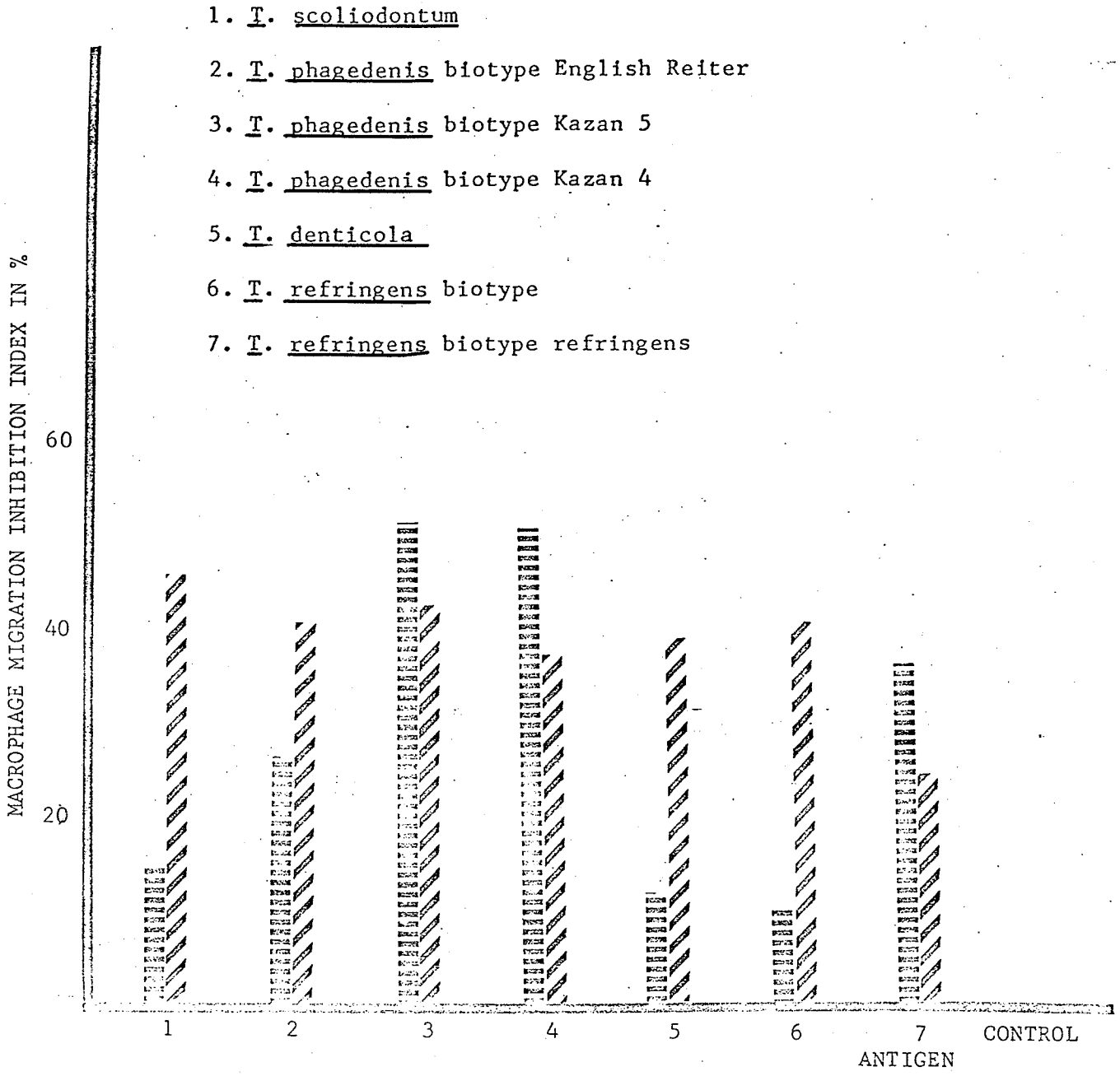


Fig.15 Macrophage migration inhibition indices of animals sensitized with T.phagedenis biotype Kazan 4.

after the 20th day intradermal challenge

after the 30th day intradermal challenge

The average areas of migration of macrophage from sensitized animals in the presence of different antigens were summarized in Table XXX. It can be seen that the average area of migration of macrophage in the presen of T.scoliodontum was 5.797 in^2. The other averages were: T.phagedenis biotype English Reiter (5.08 in^2), T.phagedenis biotype Kazan 5 (5.456 in^2 T.phagedenis biotype Kazan 4 (4.867 in^2), T.denticola (5.337 in^2), T.refringens biotype Nichols (6.945 in^2), T.refringens biotype refringens (6.354 in^2), and 7.748 in^2 without antigen. The homologous antigen, T. phagedenis biotype Kazan 5 gave the smallest area of migration, therefore, it is the most potent antigen to induce cellular reactivity.

(V) T. denticola

The MMI tests were carried out on animals sensitized with T. denticola and after the 12th, 20th, and 30th day skin tests. Figure 16 showed the MMI indices in the presence of different antigens. It can be seen that only the homologous antigen, T. denticola showed significant inhibition of macrophage from sensitized animals (index greater than 20%) after the 12th and 30th day skin tests, that is 30% and 22% respectively. The other antigens failed to show significant inhibition of macrophage migration and their indices were less than 20%.

The average areas of migration of macrophage in the presence of different antigens were compared and listed in Table XXXVI. The homologous antigen, T.denticola provoked the smallest area of migration in having an average area of 2.536 in^2, therefore, it is the result of the highest degree of inhibtion of macrophage migration. The average areas evoked by other antigens were: 3.252 in^2 (T.scoliodontum), 2.949 in^2 (T.phagedenis

TABLE XXX

THE COMPARISONS OF THE AREA OF MACROPHAGE MIGRATION FROM ANIMAL SENSITIZED WITH T.PHAGEDENIS BIOTYPE KAZAN 4

STR	CHA	N	MEAN	S.E.SQRD	S.E.
4	1	12	5.797	0.061	0.247
4	2	12	5.080	0.061	0.247
4	3	12	5.456	0.061	0.247
4	4	12	4.867	0.061	0.247
4	5	12	5.337	0.061	0.247
4	6	12	6.945	0.061	0.247
4	7	12	6.354	0.061	0.247
4	8	12	7.748	0.061	0.247

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

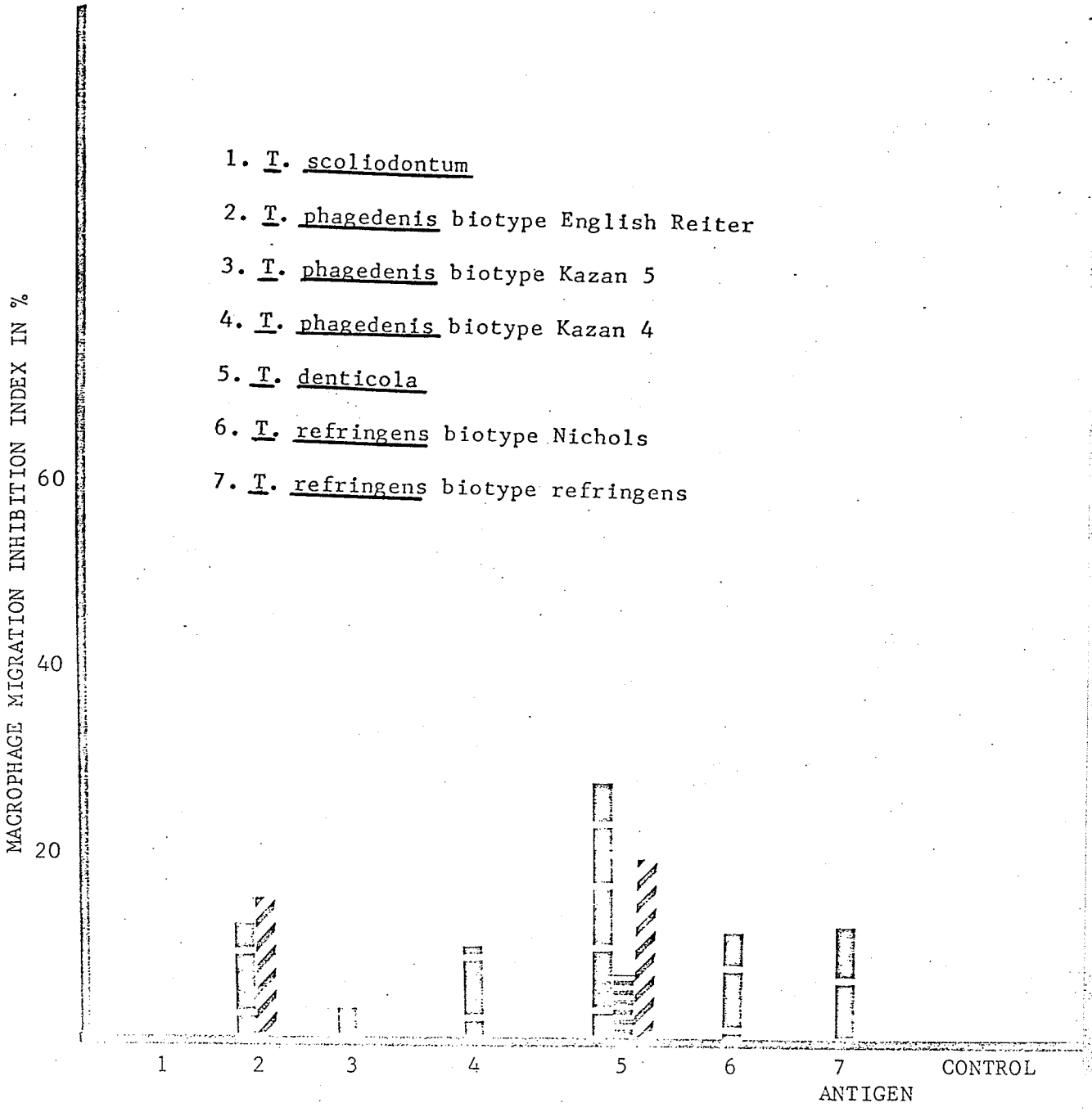


Fig.16 Macrophage migration inhibition indices of animals sensitized with T.denticola.

after the 12th day intradermal challenge

after the 20th day intradermal challenge

after the 30th day intradermal challenge

biotype English Reiter), 3.272 in^2 (T.phagedenis biotype Kazan 5), 3.122 i (T.refringens biotype Kazan 4), 3.209 in^2 (without antigen).

(VI) T.refringens biotype Nichols

Animals sensitized with T.refringens biotype Nichols and macrophage migration inhibition tests were carried out after the 12th, 20th, and 30th day intradermal challenge. It was shown in figure 17 that T. scoliodontum started with a MMI index of 8% after the 12th day skin test, 19% after the 20th day skin test, and 81% after the 30th day skin test. T.phagedenis biotype English Reiter failed to inhibit the migration of macrophage after the 12th day skin test but came up with an MMI index of 11% after the 20th day skin test and 35% after the 30th day skin test. Again, T.phagedenis biotype Kazan 5 began with an index of 17% after the 12th day skin test, and the index became insignificant after the 20th day skin test, but the index became 23% after the 30th day skin test. T.phagedenis biotype Kazan 4 had an index of 25% after the 12th day skin test, 23% after the 20th day skin test, and 81% after the 30th day skin test. T.denticola gave rise to 10% ,43%, and 56% after the 12th, 20th, and 30th day intradermal challenge, respectively. T.refringens biotype Nichols and T.refringens biotype refringens both started with an index of 28% after the 12th day skin test, and the former showed an index of 47% after the 20th day skin test and 58% after the 30th day skin test while the latter had an index of 32% and 74% after the 20th and 30th day skin test, respectively.

The results were expressed as the average areas of migration of macrophage in the presence of different antigens and listed in Table XXXII. The areas in an increasing order (i.e., decreasing order in degree of inhibition of migration) are the following: 2.545 in^2 (T.phagedenis biotype

TABLE XXXI

THE COMPARISONS OF THE AREA OF MIGRATION OF MACROPHAGE FROM ANIMALS SENSITIZED WITH T.DENTICOLA

STR	CHA	N	MEAN	S.E.SQRD	S.E.
5	1	12	3.252	0.061	0.247
5	2	12	2.949	0.061	0.247
5	3	12	3.272	0.061	0.247
5	4	12	3.122	0.061	0.247
5	5	12	2.536	0.061	0.247
5	6	12	3.209	0.061	0.247
5	7	12	3.141	0.061	0.247
5	8	12	3.129	0.061	0.247

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

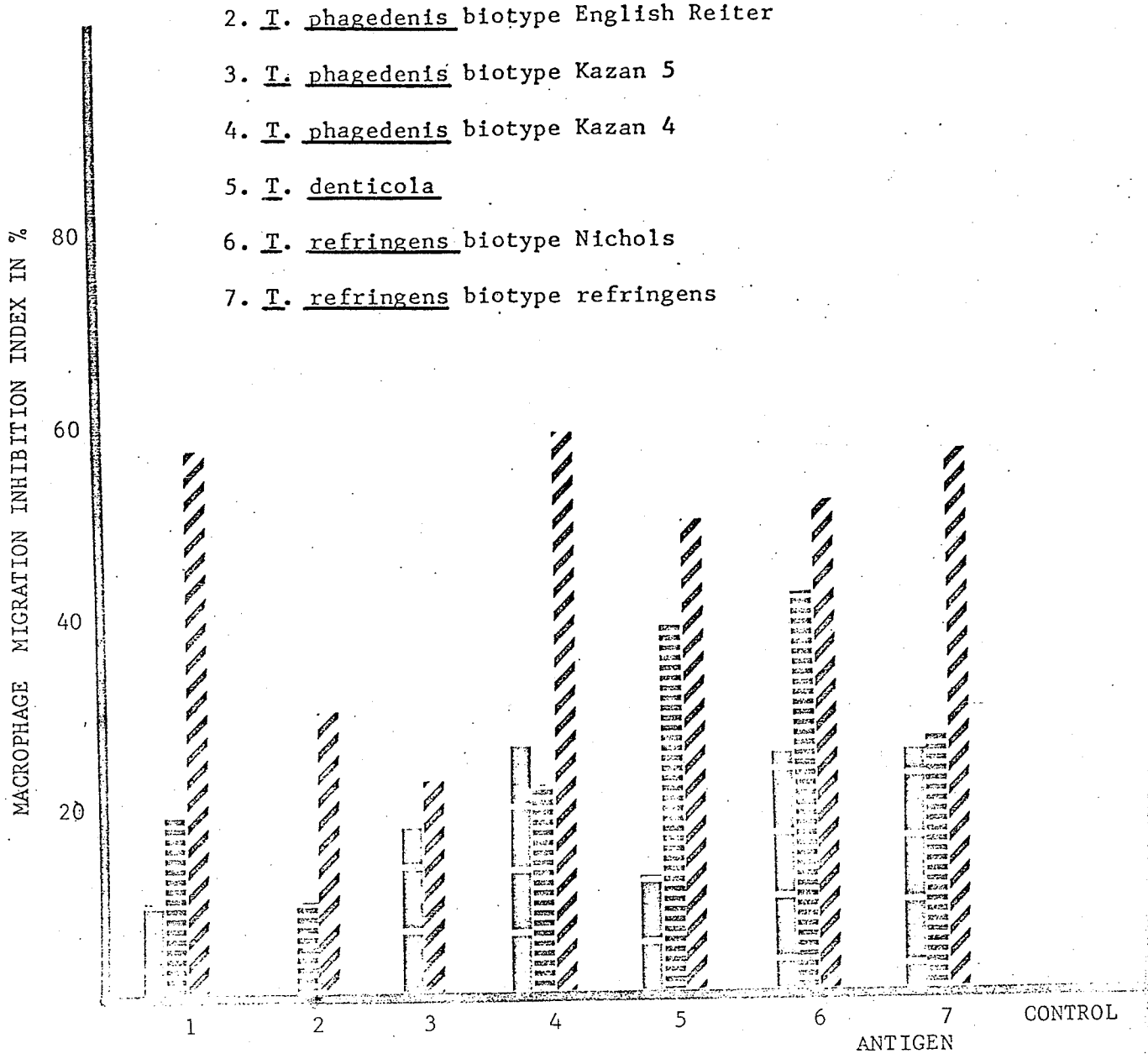


Fig.17 Macrophage migration inhibition indices of animals sensitized with T.refringens biotype Nichols.

after the 12th day intradermal challenge

after the 20th day intradermal challenge

after the 30th day intradermal challenge

TABLE XXXII

THE COMPARISONS OF THE AREA OF MIGRATION OF MACROPHAGE FROM ANIMALS SENSITIZED WITH T.REFRINGENS BIOTYPE NICHOLS

STR	CHA	N	MEAN	S.E.SQRD	S.E.
6	1	12	2.762	0.061	0.247
6	2	12	5.179	0.061	0.247
6	3	12	5.743	0.061	0.247
6	4	12	2.545	0.061	0.247
6	5	12	3.751	0.061	0.247
6	6	12	3.453	0.061	0.247
6	7	12	2.807	0.061	0.247
6	8	12	7.016	0.061	0.247

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E.:standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

Kazan 4), 2.762 in^2 (T.scoliodontum), 2.807 in^2 (T.refringens biotype refringens), 3.453 in^2 (T.refringens biotype Nichols), 3.751 in^2 (T.denticola), 5.179 in^2 (T.phagedenis biotype English Reiter), 5.743 in^2 (T.phagedenis biotype Kazan 5), and 7.018 in^2 for the control without antigen.

(VII) T.refringens biotype refringens

Animals sensitized with T.refringens biotype refringens and MMI test were carried out after the 12th, 20th, and 30th day skin test.

The macrophage migration inhibition indices of different antigens were shown in figure 18. It can be seen that only T. phagedenis biotype English Reiter (MMI index 24%), T. phagedenis biotype Kazan 5 (MMI index 22%), and T. refringens biotype Nichols (MMI index 28%) showed significant inhibition of migration of macrophage after the 12th day skin test. The indices of other antigens were insignificant and less than 20%. Finally, all antigens failed to show significant inhibition of the migration of macrophage after the 20th and 30th day skin tests.

The average areas of migration of macrophage in the presence of different antigens were listed in Table XXXIII and the results were as follows: T.scoliodontum (3.492 in^2), T.phagedenis biotype English Reiter (3.037 in^2), T.phagedenis biotype Kazan 5 (3.001 in^2), T.phagedenis biotype Kazan 4 (2.969 in^2), T.denticola (3.316 in^2), T.refringens biotype Nichols (3.202 in^2), T.refringens biotype refringens (3.202 in^2), and the control without antigen (3.151 in^2).

The mutual relationships among treponemal antigens shown in the macrophage migration inhibition test

The results obtained from the macrophage migration inhibtion tests which were carried out after the 20th and 30th day intradermal challenge were used for the calculation of the mutual relationships among treponemal antigens.

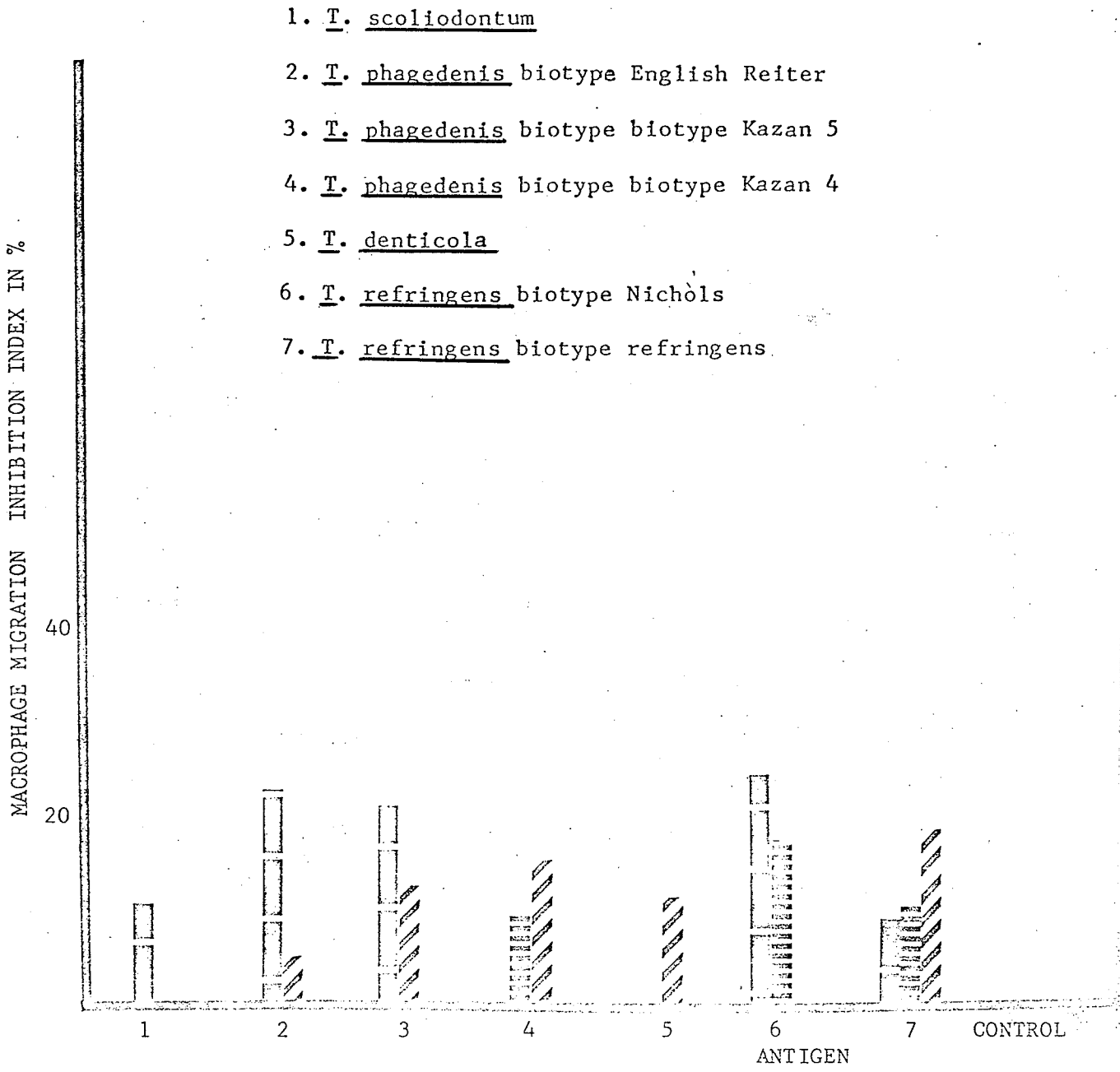


Fig.18 Macrophage migration inhibition indices of animals sensitized with T.refringens biotype refringens.

after the 12th day intradermal challenge

after the 20th day intradermal challenge

after the 30th day intradermal challenge

TABLE XXXIII

THE COMPARISONS OF THE AREA OF MIGRATION OF MACROPHAGE FROM ANIMALS SENSITIZED WITH T.REFRINGENS BIOTYPE REFRINGENS

STR	CHA	N	MEAN	S.E.SQRD	S.E.
7	1	12	3.492	0.061	0.247
7	2	12	3.037	0.061	0.247
7	3	12	3.001	0.061	0.247
7	4	12	2.969	0.061	0.247
7	5	12	3.316	0.061	0.247
7	6	12	2.672	0.061	0.247
7	7	12	3.202	0.061	0.247
7	8	12	3.151	0.061	0.247

STR: strains for sensitization

CHA: challenging antigens

N: number of readings

S.E.SQRD: standard error of square

S.E.: standard error

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens
8. saline as the control

The Duncan's New Multiple-Range test was used for observing the significant relationships among the antigens. A significant student's t test range of 5% was chosen. Each antigen was in turn assumed to be one hundred percent and a simple percentage calculation was used to obtain the comparative percentage of each antigen. Seventy percent was selected as the minimal percentage to show the positive relationship. The results were summarized as the following:

1) The mutual relationships among treponemal antigens in the macrophage migration tests after the 20th day skin test

The averages of area of migration of macrophage in the presence of different treponemal antigens after the 20th day skin test were: T. scoliodontum (2.421 in^2), T.phagedenis biotype English Reiter (4.289 in^2), T.phagedenis biotype Kazan 5 (3.712 in^2), T.phagedenis biotype Kazan 4 (1.245 in^2), T.denticola (3.29 in^2), T.refringens biotype Nichols (2.378 in^2), and T.refringens biotype refringens (6.088 in^2). These averages of area of migration were used for the Duncan's New Mutiple-Range test and for the comparative percentage calculation. The results showed that there was no significant difference ($\alpha = 0.05$) between T.phagedenis biotype Kazan 5 and T.phagedenis biotype English Reiter (comparative percentage 86 and 85, see Table XXXIV), T.denticola and T.scoliodontum (comparative percentage 65 and 73), T.denticola and T.phagedenis biotype English Reiter (comparative percentage 76 and 70), T.denticola and T.phagedenis biotype Kazan 5 (comparative percentage 88 and 88 (, T.refringens biotype Nichols and T.scoliodontum (comparative percentage 98 and 99), and T.refringens biotype Nichols and T.denticola (comparative percentage 72 and 62). The

other treponemal antigens did not show any mutual relationships according to the Duncan's New Multiple-Range test and their corresponding comparatibe percentages were less than 70%.

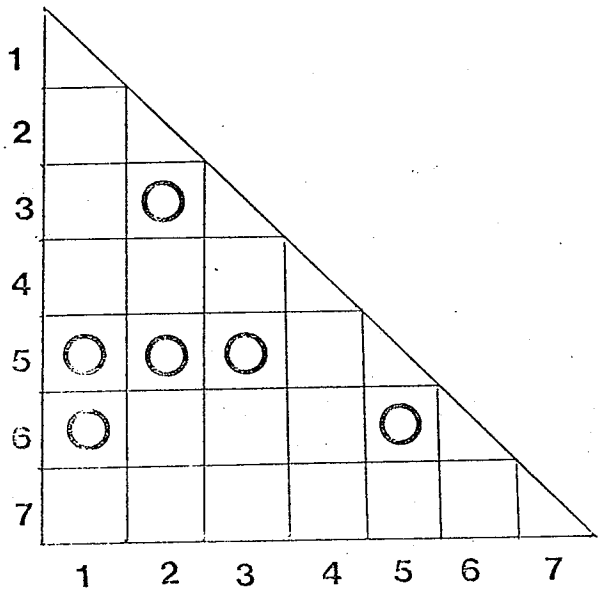
2) The mutual relationships among treponemal antigens in the macrophage migration inhibition tests after the 30th day intradermal challenge

The average of area of migration of macrophage in the presence of different treponemal antigens after the 30th day skin test were: T. scoliodontum (2.532 in^2), T. phagedenis biotype English Reiter (5.757 in^2), T. phagedenis biotype Kazan 5 (3.705 in^2), T. phagedenis biotype Kazan 4 (11.046 in^2), T. denticola (2.943 in^2), T. refringens biotype Nichols (7.188 in^2), and T. refringens biotype refringens (1.230 in^2). From the Duncan's New Multiple-Range test and the comparative percentage calculation, it showed that there was no significant different (X =0.05) between T. denticola and T. scoliodontum (comparative percentage 84 and 86, see Table XXXV), and T. denticola and T. phagedenis biotype Kazan 5 (comparative percentage 79 and 75).

It is very interesting that T. refringens biotype Nichols and T. phagedenis biotype English Reiter did not show any mutual relationship according to the Duncan's New Multiple-Range test but their corresponding comparative percentage were 76 and 80. The other treponemal antigens did not show any mutual relationships and their corresponding comparative percentages were less than 70%.

TABLE XXXIV

THE DUNCAN'S NEW MULTIPLE-RANGE TEST AND COMPARATIVE PERCENTAGE OF THE MACROPHAGE MIGRATION INHIBITION AFTER THE 20TH DAY INTRADERMAL CHALLENGE



a. The Duncan's New Multiple-Range test.

○: There is no significant difference form each other ($\alpha = 0.05$).

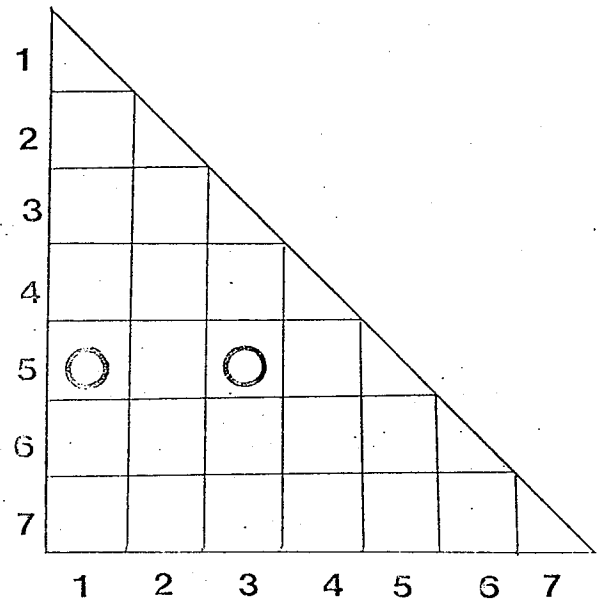
	1	2	3	4	5	6	7
1	100	56	65	7	73	99	39
2	23	100	85	44	70	20	70
3	47	86	100	2	88	44	60
4	51	29	33	100	37	52	20
5	65	76	88	36	100	62	54
6	98	55	64	8	72	100	39
7	51	59	36	12	15	44	100

b. The comparative percentage of treponemal antigens, percentage higer than 70 were positive for mutual relationship.

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens

TABLE XXXV

THE DUNCAN'S NEW MULTIPLE-RANGE TEST AND COMPARATIVE PERCENTAGE OF THE MACROPHAGE MIGRATION INHIBITION AFTER THE 30TH DAY INTRADERMAL CHALLENGE



a. The Duncan's New Multiple-Range test.

○: There is no significant difference from each other ($\alpha = 0.05$).

	1	2	3	4	5	6	7
1	100	43	68	22	86	36	5
2	27	100	45	52	5	80	32
3	54	64	100	33	75	51	1
4	36	9	2	100	25	47	2
5	84	51	79	26	100	40	39
6	17	74	6	65	44	100	16
7	48	21	33	11	41	17	100

b. The comparative percentage of treponemal antigens, percentage higer than 70 were postive for mutual relationship.

1. T.scoliodontum
2. T.phagedenis biotype English Reiter
3. T.phagedenis biotype Kazan 5
4. T.phagedenis biotype Kazan 4
5. T.denticola
6. T.refringens biotype Nichols
7. T.refringens biotype refringens

III. Macrophage migration inhibition as an in vitro correlate of delayed skin test

Figure 19 presented data of macrophage migration inhibtion performed with treponemal antigens (T.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) in relation to the measurement (the longest axis $\times$ the shortest axis of erythema and induration of skin) of the corresponding trepronemal antigens skin reaction (24 hours after intradermal challenge) in guinea pigs sensitized with homologous treponemal antigens. The data were obtained from 84 guinea pigs divided into seven groups. Twelve guinea pigs were used for each treponemal antigen. The macrophage migration inhibition were regarded as positive when the macrophage migration inhibition indices were more than 20%, and negative, when it was less than 20%. The skin reactions were considered positive when the measurements of the erythema and induration were 40 mm^2 or more; negative when measurements were less than 40 mm^2. It was found that 54.76% ($\frac{46}{84} \times 100\%$) of the data were under the positive macrophage migration inhibition (MMI) and skin reaction, 29.77% ($\frac{25}{84} \times 100\%$ gave positive skin reaction but negative MMI, 7.14% ($\frac{6}{84} \times 100\%$) showed positive MMI but negative skin reaction, and finally, 8.33% ($\frac{7}{84} \times 100\%$) of the results were negative MMI and skin reaction. The positive skin reactions were accompanied in most of the cases by postive MMI. Whereas positive skin reactions frequently associated with negative MMI.

The data showed positive MMI and positive skin reaction were as follows: 6/12 for T. scoliodontum; 4/12 (T. phagedenis biotype English Reiter); 9/12 (T. phagedenis biotype Kazan 5); 8/12 (T. phagedenis biotype Kazan 4); 3/12 (T. refringens biotype refringens); 4/12 (T. denticola); and 12/12 (T. refringens biotype Nichols).

Figure 20 showed the macrophage migration inhibition tests in the presence of heterologous antigens and the skin reactions induced by the same antigens. Seventy-two readings (six heterologous antigens for twelve guinea pigs) were obtained for each group and seven groups amounted to 504 readings.

It was found that 21.43% ($\frac{108}{504}$ X 100%) of the data obtained from heterologous antigens showed positive MMI and skin reactions; 30.75% ($\frac{155}{504}$ X 100) were negative MMI and skin reactions; 30.55% ($\frac{154}{504}$ X 100%) were positive skin reaction but negative MMI; and 17.27% ($\frac{87}{504}$ X 100%) were positive MMI but negaitve skin reaction. It can be concluded that the positive skin reactions were accompanied in most cases by negative MMI and positive MMI were frequently associated with negative skin reactions.

The data of positive skin reaction and MMI against heterologous antigens were the following: 16/72 (sensitized with T. scoliodontum); 21/72 (sensitized with T. phagedenis biotype English Reiter); 5/72 (T. phagedenis biotype Kazan 5); 17/22 (T. phagedenis biotype Kazan 4); 4/72 (sensitized with T. denticola); 42/72 (sensitized with T. refringens biotype Nichols); and 3/72 (sensitized with T. refringens biotype refringens).

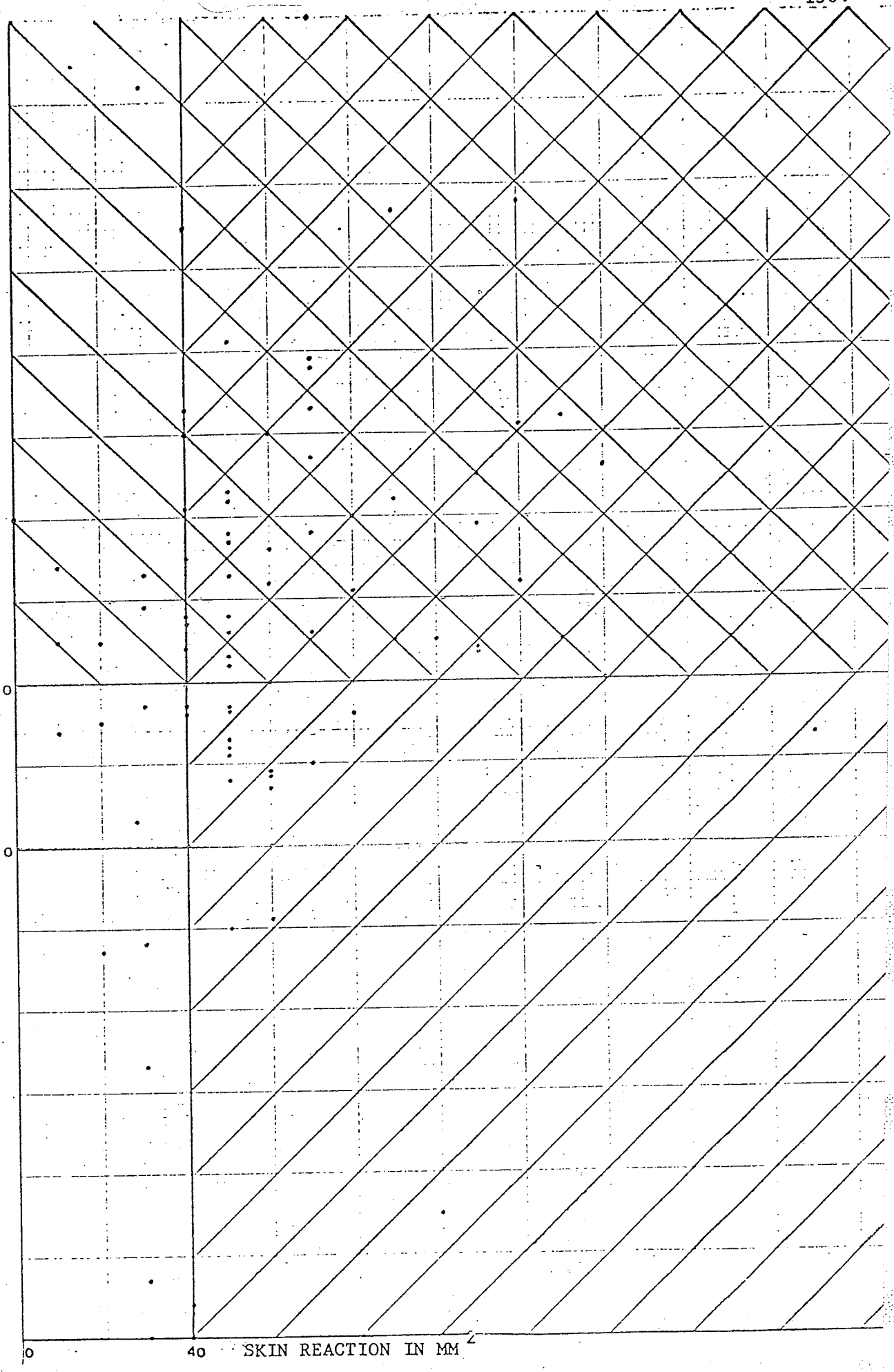

MACROPHAGE MIGRATION INHIBITION INDEX IN %
20
0
0
40
SKIN REACTION IN MM 2

Fig. 19

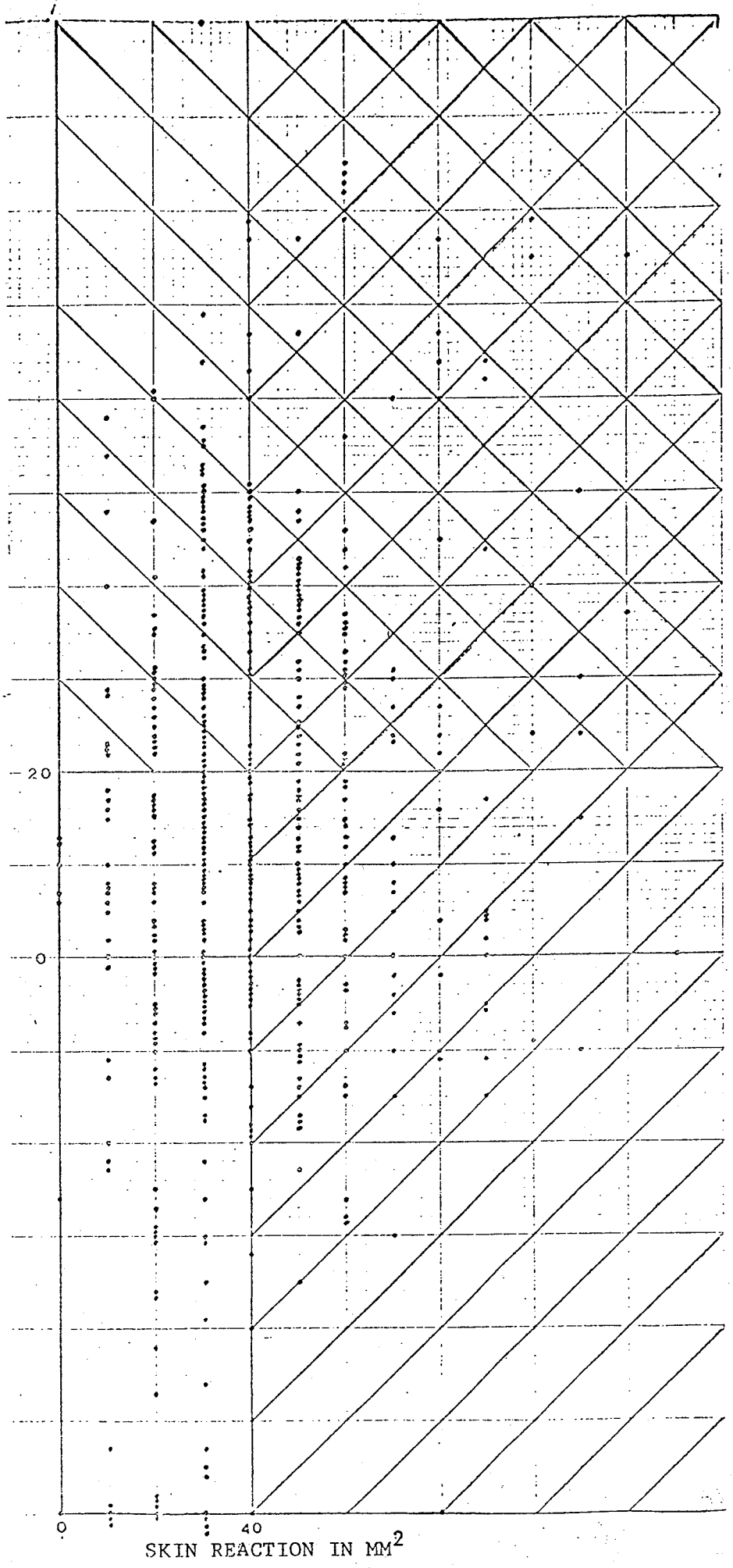

MACROPHAGE MIGRATION INHIBITION INDEX IN %
20
0
0
40
SKIN REACTION IN MM2

Fig. 20

DISCUSSION

In 1953, D'alwssandro et al.,[68] were the first to demonstrate a protein antigen in Treponema reiter in studying the complement fixation reaction which occurs when this spirochete or its extracts are placed in presence of syphilitic serum. This antigen has been identified by diffusion precipitation test (Pillot, 1960)[69] and was also demonstrated in Treponema pallidum (Dardononi and Censuales, 1957;[70] Cannefax and Garson, 1959 [71]), in T. zuelzerae and in saprophytic treponemes isolated from genital sources (Duponey, 1963[54]). Although this antigen has not yet been obtained in a purified atate, it is possible to consider that the antigen is present in all the representatives of the Treponema genus. Later, Mac Leod, Garson (1962 [72]) and Miller (1966[73]) were able to demonstrate the existence of specific protein antigens from a series of experiments. The investigators reported that anti-T. pallidum antiserum, or syphilitic sera having undergone an exhaustion series permitting elimination of reagin and the group antiprotein fix complement with a thermolabile fraction of T. pallidum. All these suggested that the protein antigens of treponemes may play an important role in the antigenic and immunogenic activities. Therefore, the cytoplasmic soluble antigens of treponemes (T.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) were used for this studies of cell-mediated immune response to treponemal antigens. An in vivo test(delayed hypersensitivity skin test) and an in vitro test (macrophage migration inhibition test) were applied for the studies.

Several of the classical criteria for demonstrating delayed hypersensitivity were fulfilled in this experiment. First, the skin reaction was

delayed in onset and remainedfor several days, in some cases a reaction was still visible after 72 hours. Second, histological reaction was compatible with that described for delayed hypersensitivity by having an early acute inflammatory response with edema and polymorph infiltration was soon followed by accumulation of macrophage and lymphocytes (Fig. 9a to 9d). The predominant reaction is therefore mononuclear in contrast to the exudative response of anaphylaxis and the polymorph accumulation of the Arthus reaction. In severe reaction, when challenged with homologous antigens, necrosis of the skin was found (Fig. 9e).

The macrophage migration inhibition test, as first described by George and Vaughan[8], and further used by David et al.[9], Bloom and Bennet[10, 11], and others, is considered to be an in vitro correlate of delayed hypersensitivity as measured by skin test. In the present work, the macrophage migration inhibitions were regarded as positive when the macrophage migration inhibition indices were more than 20% while the skin reactions were considered positive when the measurements of induration were more than 40 mm^2. When sensitized animals challenged with homologous treponemal antigens, 54.76% of the results showed positive skin reactions and positive macrophage migration inhbition while only a percentage of 21.43 was shown when heterologous treponemal antigens were used. The results of the macrophage migration inhibition tests did not show strong correlation with those of skin test; but the treponemal antigens were capable of eliciting cellular immune reaction in the animals in terms of delayed hypersensitivity and inhibitions of migration of macrophages. The higher percentage of correlation in homologous

treponemal antigens indicated the specificities of the antigens and the correlation of the neterologous treponemal antigens showed the cross-reaction among the antigens.

The variation of macrophage migration inhibition as an in vitro correlate of delayed skin reaction was dependent on the test animals and the macrophage migration inhibition test perfromed. The varied immune mechanisms of each animal, the condition in carrying out the macrophage migration inhibition test (i.e., pH, temperature, and the metabolic activities of macrophages) can effect the results of the tests. Furthermore, there is only a single mediator, the migration inhibiting factor (MIF) of known chemical properties may be considered responsible for the macrophage migration inhibition while in the delayed type skin reactions, a great number of mediators may play a role[74,75] The quantitative or qualitative change of these substances may alter the manifestation of skin reaction even in the case of developed cellular immunity. Finally, the positivity of macrophage migration inhibition test seems to depend on the availability of a very few sensitized lymphocytes[76], whereas skin test positivity needs the presence of other mononuclear cells.

The range of immunological specificity of the treponemal antigens evoking cell-mediated immunity can be demonstrated from the degree of sensitization among strains and the degree of reaction in the presence of different treponemal antigens.

According to the results of skin tests, the degree of sensitizati among treponemal strains in decreasing order were as follows (Table XIII): 1) T.refringens biotype Nichols, 2) T.phagedenis biotype English Reiter, 3) T.denticola, 4) T.phagedenis biotype Kazan 4, 5) T.phagedenis biotype

Kazan 5, 6) T. refringens biotype refringens, and 7) T. scoliodontum. The degrees of skin reaction to different challenging antigens in an decreasing order were (Table XIV): 1) T. refringens biotype Nichols, 2) T. refringens biotype refringens, 3) T. denticola, 4) T. phagedenis biotype Kazan 4, 5) T. refringens biotype English Reiter, 6) T. refringens biotype Kazan 5, 7) T. scoliodontum, 8) control. It can be seen that the T. refringens biotype Nichols is the strongest antigen to induce skin reactions on sensitized animals. T. scoliodontum is the weakest antigen for sensitization and for intradermal challenge. Some antigens are only good for sensitization while others are only good for intradermal challenge.

Since delayed hypersensitivity skin tests and macrophage migration inhibition test are two independent assays, the ranges of immunological specificity of the treponemal antigens obtained from both tests are different.

The degrees of sensitization among strains for macrophage migration tests in decreasing order were (Table XXV): 1) T. scoliodontum, 2) T. denticola, 3) T. refringens biotype refringens, 4) T. phagedenis biotype Kazan 5, 5) T. phagedenis biotype English Reiter, 6) T. refringens biotype Nichols, and 7) T. phagedenis biotype Kazan 4. T. scoliodontum is the most potent antigen to inhibit the migration of macrophages from sensitized animals. There is of no significant difference for the inhibition of migration of macrophage in the presence of different treponemal antigens (Table XVI).

Previous studies of the treponemes by use of agglutination, complement fixation, and gel-diffusion methods, have revealed immunological data of taxonomic importance. Meyer and Hunter (1966)[45] demonstrated the antigenic relationships of 14 treponemes by immunofluorescence. The investigators divided the 14 treponemes into five categories based on shared group-specific antigens. Serogroup I: Reiter, English Reiter, Kazan, Kazan 2, 4, 5, and 8; Serogroup II: Nichols and Noguchi; Serogroup III: three oral treponemes, MRB, FM, and N-39 Serogroup IV: I. vincentii; Serogroup V: Treponema zuelzerae. The five serogroups apparently are related by an immunofluorescent common antigen.

In these studies, the mutual relationships among treponemes were obtained from the Duncan's New Multiple-Range test and from a simple percentage calculation.

The mutual relationships among treponemes obtained from skin tests can be divided into two groups. Group I: T.phagedenis biotype English Reiter, T.phagedenis biotype Kazan 4, and T.denticola. Group II; T.scoliodontum, T.phagedenis biotype Kazan 5, and T.refringens biotype refringens. These two groups were able to demonstrate their relationships on the 20th and 30th day intradermal challenge while other treponemes only showed their relationships on the 20th or 30th day intradermal challenge (Table XXII and Table XXIII).

It was found that only T.scoliodontum, T.phagedenis biotype Kazan 5, and T.denticola showed their relationships when macrophage migration inhibition test was performed. These relationships were also found after the 20th and 30th day intradermal challenge. However, other treponemes showed their relationships after the 20th day intradermal challenge but the relationships subsided after the 30th day intradermal challenge (Table XXXIV and Table XXXV).

In conclusion, the period of sensitization for developing the immune response and the decrease of immune response with time can affect the finding of the mutual relationships among treponemes.

Although skin testing with Treponema pallidum as an antigen has been used in human beings,[77] partially purified T.pallidum failed to induce skin reaction on rabbits sensitized with live T.pallidum in this experiment. This finding has confirmed the observations of Schell et al (1975)[78] and others (Turner, Thomas B., personal communication) that skin-testing in rabbits with T.pallidum antigen gave a meager response which is difficult to interpret; this may be related to the form of the treponemal antigen or to the nature of skin testing for delayed hypersensitivity in the rabbit. On the contrary, the non-pathogenic treponemal antigens provoked erythema and induration skin reactions on the infected rabbits. This may be related to the common antigen shared by treponemes.

It has become increasingly apparent that cell-mediated immunity plays an important role in the host response to the infection with T.pallidum. Further investigations are needed to determine parts of treponemes and its chemical and antigenic composition in term of cell-mediated immunity, and particularly those relevent to Treponema pallidum and syphilis.

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