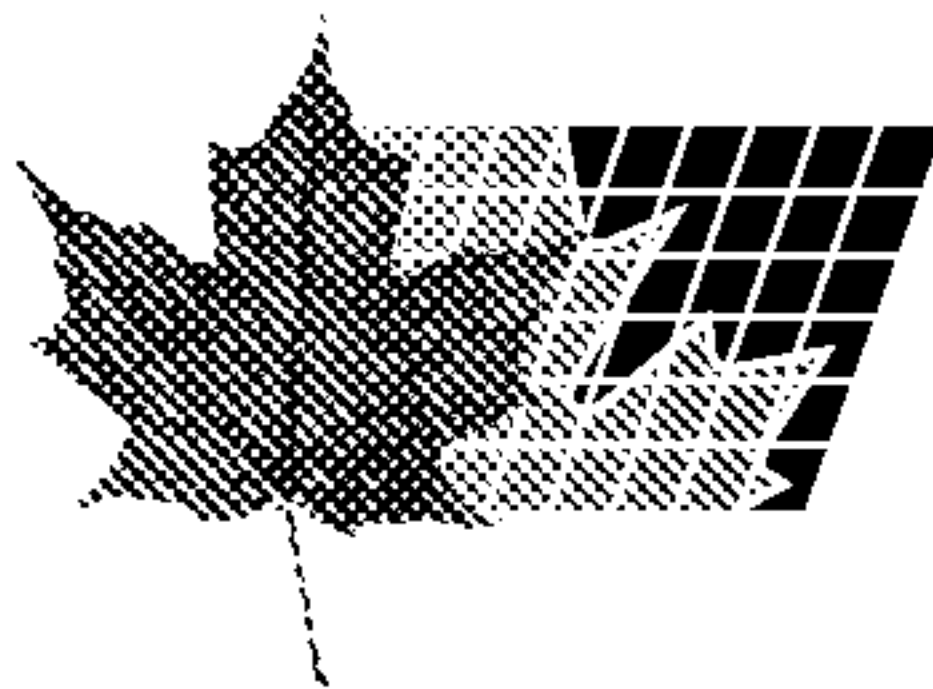




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(54) **VACCINS CONTRE LES INFECTIONS A CHLAMYDIAE ET
LEUR PROCEDE**

(54) **CHLAMYDIA VACCINES AND PROCESS**

(57) A genus specific chlamydia oral or injectable vaccine is provided which comprises an anti-idiotypic antibody capable of producing in an animal an anti-idiotypic antibody or Fab fragment thereof enclosed in microspheres formed of a pharmacologically acceptable polymer is capable of producing in an animal an anti-anti-idiotypic immune response (serum antibody, secretory antibody or T-cell response) which recognizes a glycolipid exoantigen (GLXA) of chlamydia. The oral or injectable vaccine is produced from an idiotypic antibody to GLXA which, in turn, is utilized to produce the anti-idiotypic antibody.



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(54) Title: CHLAMYDIA VACCINES AND PROCESS (57) Abstract A genus specific chlamydia oral or injectable vaccine is provided which comprises an anti-idiotypic antibody capable of producing in an animal an anti-idiotypic antibody or Fab fragment thereof enclosed in microspheres formed of a pharmacologically acceptable polymer is capable of producing in an animal an anti-anti-idiotypic immune response (serum antibody, secretory antibody or T-cell response) which recognizes a glycolipid exoantigen (GLXA) of chlamydia. The oral or injectable vaccine is produced from an idiotypic antibody to GLXA which, in turn, is utilized to produce the anti-idiotypic antibody.		

BACKGROUND OF THE INVENTION

This invention relates to vaccines including oral vaccines against chlamydial antigens, and a process for making the vaccine.

Chlamydial infection is a diverse group of conjunctival, genital, respiratory, and neonatal infections occurring primarily on mucosal surfaces. The etiologic agent of the infection is an obligate intracellular bacterial parasite of eukaryotic cells, chlamydia. There are three genetically different species in this genus, with certain similarities in morphology, intracellular developmental cycle and antigenic responses: Chlamydia trachomatis, Chlamydia psittaci and Chlamydia pneumoniae.

The infection by C.trachomatis is limited to humans. Fifteen serovars are differentiated based on the antigenic variations of the major outer membrane protein (MOMP) Grayston and Wang, J. Infect. Dis., 132:87, 1975), Serotypes D-K, are the most common cause of sexually transmitted venereal diseases. Conservatively, more than 4 million cases of chlamydial sexual infections occur each year in the United States making it more prevalent than all other sexually transmitted diseases combined. The diseases include nongonococcal urethritis, mucopurulent cervicitis, acute epididymitis, ectopic pregnancy and pelvic inflammatory disease (PID, endometritis, salpingitis, parametritis and/or peritonitis). The infection in women can be quite damaging: Among 250,000 cases of pelvic inflammation

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diseases caused by this organism in the U.S. each year, 10% lead to infertility. When infants are born to chlamydia-infected mothers, they are at risk of developing inclusion conjunctivitis and pneumonia. C. trachomatis serovars A, B, Ba, and C cause trachoma, an infection of conjunctival epithelial cells. The chronic and secondary infections induce the infiltration of subepithelial lymphocytes, forming follicles and the invasion of fibroblasts and blood vessels to

the cornea, leading to blindness. On the other hand, the formation of the scar and malformation of the eyelid, causing trichiasis' constant scraping of the cornea by the eyelash can also lead to corneal opacification and blindness. There are approximately 500 million trachoma cases in the world, and between 7 and 9 million are now blind because of its complications making it the world's leading cause of preventable blindness. The prevalence of active trachoma is high in early age. There are 80 million children in need of treatment. It has been an enormously important health problem in the Middle East, North Africa, South Asia and North India.

C. psittaci mainly affects animals and birds. It had, and still has a great economic impact in dairy, wool and meat industries. There are 9 serovars from mammalian species, 7 serovars from avian species and 2 biovars from koala bears. Mammalian serovar 1, 2, 3, and 9 infect cattle and sheep, causing a wide range of disorders from placenta and fetus infection and other reproductive problems, including polyarthritis-polysitis, encephalomyelitis, conjunctivitis as well as intestinal infections. Although numerous attempts have been made to produce vaccines, only modest success has been achieved (Schnorr, J. Am. Vet. Med. Assoc. 195:1548, 1989). Serovars 4, 5, and 6 are the causes of abortions, pneumonia and polyarthritis in porcine species. Serovar 7 represents chlamydial strains of feline conjunctivitis, rhinitis and pneumonitis and serovar 8 includes guinea pig inclusion conjunctivitis. The avian strains often cause human infection in bird handlers and poultry processing workers.

C. pneumoniae is a newly identified species. To date, one serovar has been identified, TWAR (Grayston, Proceedings of the Seventh International Symposium on Human Chlamydial Infections, Pg. 89, 1990). Current evidence suggests that C. pneumoniae is a primary human pathogen that is transmitted from human to human and causes about 10-20% of community acquired pneumonia in adults. It has become the main causative agent of human respiratory diseases such as pneumonia, bronchitis, pharyngitis, and sinusitis and a possible

agent in reactive arthritis. Epidemics have occurred in hospitals, in the military and families. The serological finding from many countries have shown that 50-55% of adults with antibody against TWAR antigen are specific for C. pnueunoniae. It is the major bacterial cause of illness in newborn. The infection to elderly persons and those with chronic diseases may cause serious illness or even death.

The pathogenicity of chlamydial infection is not well understood. It is long known that different individuals infected by these serovars exhibit different clinical manifestations. It has been proposed that it was likely due to the variation of the host immune response. It has been shown that immunologic response to the synthetic Th/B cell epitopes in the various inbred strains of mice is different, indicating that the T helper epitope is recognized in the context of the multiple major histocompatibility complex.

The target of chlamydial invasion are typically epithelial cells of a host. It is still not certain how the chlamydial elementary bodies (EB), (a sporelike, spherical particle, about 300 nm in diameter), enter the host cell: receptor-mediated endocytosis, and/or non-specific high affinity absorption. It has been reported that two proteins, 18 and 32 kD of C. trachomatis bind to Hela 299 cell membrane preparations. Recently, another heat-labile protein membrane protein, 38kD, was proposed a binding to Hela cell line, suggesting a ligand like mechanism. It has also been proposed that since chlamydia have the ability to infect a wide variety of mammalian cells in vitro, there must be some adherence mechanism for the establishment of the infection. The major outer membrane protein was proposed as such an adhesion. Recently, it has been demonstrated that a heparin sulfate-like glycosaminoglycans present on the surface of chlamydia organisms is required for attachment to host cells. The receptors on the host cells have also been studied. It was suggested that proteins, 18,000 and 31,000 kD from Hela cells are the receptors due to trypsin sensitivity for the EB specific binding. It also has been shown that C. trachomatis and C. pneumoniae bind

specifically to a lipid on Hela cells. Nuclear magnetic resonance spectroscopy analysis and atom bombardment mass spectrometry show that it was phosphatidylethanolamine (PE). At the same time ganglia-series glycolipids were found specifically bound to EBs. All those findings suggest that the mechanism of endocytosis by epithelial host cells is still a matter of uncertainty. Once the EBs enter the host cells by endocytosis, depending upon conditions, they are transformed into a metabolically active, non-infectious reticulate body (RB). The prime purpose of RBs is intracellular replication by binary fission using host metabolites. This occurs in a membrane-bounded vesicle, termed an inclusion. This inclusion (endosome) can resist the fusion with the lysosomes of host cells. Each RB eventually gives rise to one or more EBs which can initiate another infectious cycle. Host cells may be lysed by release of inclusion bodies or undamaged by exclusion body exocytosis. Surface antigens are thought to direct both phagocytosis and evasion of phagolysosomal fusion.

The treatment of chlamydial infections has relied on the administration of antibiotics. This has been proved effective in the early stages of the infection depending upon proper timing for diagnosis and screening. The problem is that the infection can be asymptomatic. Most patients don't realize its presence until it has occurred for a period of time. In the chronic stage as in the case of genital infection, it has been demonstrated that little can be done to prevent the damage of the reproductive tracts in a monkey infection model.

Vaccines employing the whole organism or sub-units of the organism have been used in an attempt to prevent chlamydia infections caused by members of the trachoma biovars. These attempts, however, have been disappointing, partially due to host hypersensitivity in reaction to the vaccines (Grayston et al, Clin. Med. J. (Republic of China) 8: 312, 1961, Wang et al, 1967, Am. J. Ophthalmol. 63: 1615, Schachter, Pathol. Immunopathol. Res. 8: 206, 1989). The pathogenesis associated with infections believed to be a process of delayed hypersensitivity. It is thought that chronic inflammation resulting from repeated

reinfection of humans have an important role in the conjunctival infiltration, blinding sequelae of trachoma and scarring of the fallopian tubes which result in infertility and ectopic pregnancy. The surface antigens of elementary bodies have been the focus of research attempting to identify a protective antigen.

Surface components of chlamydia actively interact with host cells and with the host's immune system. They are believed to account for the attachment, endocytosis and the immune response, but the exact nature and regulation of these interactions has not yet been fully identified. Several distinct antigenic components of C. trachomatis, C. psittaci and C. pneumonia have been investigated including the identification, characterization and function in chlamydial infection. Moreover, it is of importance to determine the mechanism of infection and determine the protective antigens. Surface exposed antigens are the main targets of much research since they are accessible to the immune or other defense systems. The antigens most actively investigated include major outer membrane proteins (MOMP) chlamydial lipopolysaccharide, 60-kD heat shock protein (HSPO 60) adhesions and a glycolipid exoantigen termed the exoantigen (GLXA).

In the outer membrane of chlamydia there are three cysteine-rich proteins 57, 40, and 12.5 kD which resembles the matrix proteins of gram-negative bacteria. The 57 and 12.5 kD proteins can not be found in the replicating form of the bacteria RBs. As the major outer membrane protein (MOMP), 40 kD, is abundant in both infectious EBs and RBs. In RBs, the protein could function as pore-forming proteins that permit exchange of nutrients for the reticulate bodies. Genetic and molecular characterization have shown that this protein is composed of four variable segments (VS) interspersed among five constant segments. Those variable segments are surface exposed and have the determinants of serovar, subspecies and species specificity.

The studies on immune responses to this protein are mostly carried out by immunization of animals with purified protein. In vitro neutralization experiments

have been conducted using the mixture of poly or monoclonal antibodies specific to MOMP and EBs to infect cell culture. These experiments indicate that the antibodies specific to MOMP protein or one single epitope prevent the inclusion bodies formation in cell culture. The mechanism of the neutralization does not involve inhibition of the attachment or penetration, but rather interfere with the process after internalization. Using monoclonal antibodies generated by the whole elementary bodies of serovar B, the monoclonal antibodies which recognized the immuno-accessible MOMP epitope in dot blot assays, neutralized the infectivity of organisms of monkey eyes. The protection was serovar-specific. In a later experiment by using Fab fragments of the monoclonal antibody, it has been further demonstrated that monovalent Fab neutralized the infection by preventing the attachment to Syrian hamster kidney (HaK) cells. Confirming that the protection is not due to the aggregation of bivalent IgGs. T cell epitopes of MOMP have also been investigated. T cell proliferation responses were found in splenic T cells obtained from A/J mice immunized with MOMP in the presence of overlapping synthetic peptides which represent primary sequence of serovar A MOMP. The synthetic peptides which produced T cell response correspond to surface-accessible serovar-specific epitopes located in variable domains (VD) VD I and VD IV. By using a similar approach, it was found in BALB/cByJ mice that VDIII fragment is T cell dependent. It also has been shown that by using chimeric T/B cell peptides derived from two epitopes of MOMP, one is a conserved T helper cell epitope and the other is a serovar A specific neutralizing epitope. Some mice immunized with this peptide produced high-titered serum-neutralizing antibodies, while others did not. Although MOMP has been a most intensively studied surface antigen and the neutralization antisera has been produced in experimental animals, there are still many unsolved questions regarding the immune response. For example, the neutralization of infection is serovar specific, thus, it is limited as a vaccine candidate. The neutralization is still limited to in vitro studies, and there has been no convincing in vivo protection from challenge

by immunization with an MOMP or chimeric epitopes known.

It has long been known that a chlamydia genus specific antigen was a glycolipid (Dhir et al, J. Immunol. 109: 116-122, 1972). Much later, it was found that lipopolysaccharide (LPS) was in the outer membrane of both EBs and RBs of chlamydia. It has a chemical structure similar to enterobacterial LPS of the Re chemotype (Nurminen et al, Science, 220: 1279-1281, 1983). The monoclonal antibodies prepared by immunization with EBs of serovar L2 were specifically against LPS of chlamydia, but not LPS from N. Gonorrhoeae, S. typhimurium, or E. coli. However, antibodies produced by S. typhimurium Re LPS or lipid A recognized chlamydial LPS (Caldwell and Hitchcock, Infect. Immun., 44: 306, 1984)). this demonstrated that chlamydial LPS has an unique antigenic domain compared to other gram-negative bacteria. Further characterization has shown that a chlamydia-specific domain contained in its saccharide portion, 3-deoxy-D-manno-2-octulosonic acid (DKO) with a sequence of α -kDo (2-8)- α kDo(2-4- α -kDo(kDo₃)). The 2.8 linked moiety is the structural characteristic of chlamydial LPS. Studies have also been carried out in the distribution and the relocation of LPS on the outer membrane during the developmental cycle. By immunostaining with a monoclonal antibody it was shown that LPS is loosely bound in the membrane during the developmental cycle, and not shed into media.

Chlamydial LPS was thought to be an ideal antigen for the vaccine candidate because of its abundance on the surface and its being antigenic. LPS was suspected as an important virulent determinant in the early steps of the infection and the antibodies specific to it serve some function in resolving the chlamydial infection. However, little is known concerning the biological function of LPS or the immunological response to it. It appears that the antibodies which are specific to LPS only have been useful in the diagnosis of chlamydial infection and location of LPS, but not effective in resolving an infection.

Other genus-specific chlamydial antigens are 57 to 60 and 75 kD proteins which have been identified as related to the heat shock protein (HSP) family.

This was done by comparing the sequence of the operons encoding these proteins to the groE stress response operon of E. coli or B. megaterium. The antigenic identity of 57 kD protein was confirmed by the reaction with anti-HSP-60 antiserum. The 60 kD protein elicited an ocular hypersensitivity response in immune guinea pigs, which was characterized by a predominantly mononuclear macrophage and lymphocyte cellular infiltration (Watkins, et al, Proc. Natl. Acad. Sci. USA 83: 7580, 1986, Morrison et al, S. Exp. Med. 169: 663, 1989). This was the first indication that an antigen is responsible for delayed hypersensitivity in chlamydial ocular infection. The precise involvement of this protein in stimulating immunopathogenic responses in human chlamydial diseases has not been determined. There is evidence that shows a certain percentage of sera taken from women with PID, ectopic pregnancy and tubal infertility have high anti-chlamydial antibodies reacting to chlamydial HSP-60 heat-shock protein. However, not every patient serum which has high titer to chlamydia reacts with it, indicating that either HSP-60 is not surface exposed or antigenicity is MHC restricted.

The 75-kD protein was found preferentially transcribed during heat stress of chlamydial organisms. The monospecific antibodies from rabbits raised against 75-kD protein were found to bind to the organism and neutralized the infection in vitro. It is an exposed antigen in the outer membrane.

Genus-specific glycolipid exoantigen (GLXA) was originally isolated from the supernatants of chlamydia infected cell cultures (Stuart and MacDonald, Current Microbiology, 11: 123, 1982). it has been characterized chemically, biologically and serologically in recent years.(Stuart and MacDonald, Proceedings of the Sixth International Symposium on Human Chlamydia Infections, p167, 1986, Stuart et al, Immunology, 67: 527, 1987). Mass spectrographic analysis indicated that GLXA contains polysaccharides: gulose, (not glucose), mannose and possibly galactose, while the lipid component has fatty acids of chain length C17 and C18:1. There is no KDO or lipid A found in its structure. It is produced

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and released from the infected cells during the growth cycle in vitro. Transmission electron microscopy utilizing colloidal gold-conjugated goat anti-mouse second antibody to detect the specifically bound monoclonal antibody revealed that GLXA is mostly extracellular 48 hours after the infection (Stuart et al, Immunolgy, 74: 747, 1991) which is different from that found for chlamydial LPS. Human sera from patients with clinically defined lymphogranuloma venereum (LGV) contain IgG antibodies which recognize GLXA (Stuart and MacDonald, Immunology, 68: 469, 1989), demonstrating the immunoaccessibility in the natural infection. But, there was little information on its function in the chlamydial infection and the immune response to it. The overall immunological reaction to chlamydial antigens is not well understood. It is still not known how the chlamydia evade the host immune surveillance. Antibodies found specific to chlamydial antigens in infected human patients have shown little protection for later infection. Although chlamydia mainly affects mucosal surfaces, the clinical relevance of the IgA immunity to it has not been completely described. The feasibility of chlamydia vaccine depends on producing a protective host defense which may include S-IgGA response, a cell mediated response and possibly a humoral antibody. In addition, the ability to produce large quantities of this antigen indicates a synthetic and/or chimeric antigen may be the method of choice.

Idiotypes have been intensively studied following Jerne's network theory in 1974. One of his major proposals is the self-regulation of the immune system through a network of idiotypic-anti-idiotypic interactions (Jerne, 1974). It is suggested that the idiotopes on a single antibody molecule can mimic (that is, be the "internal image") of any foreign or self epitope at the molecular level.

All idiotypes of a single immunoglobulin molecule have been found to be located on Fv (fragment variable) region by studies showing that the inhibition of binding of anti-idotypic antibodies to the idiotypic is the same between Fv and Fab(Givol, 1991). In general, anti-idotypic antibodies are divided into three types

$Ab_2 \alpha$, $Ab_2 \beta$ and $Ab_2 \epsilon$. Only $Ab_2 \beta$ binds to the complementary determining region, thus can be the internal image of the antigen. The occurrence of Ab_2 displaying internal image of properties must adhere to the following criteria; (1) binding onto Ab_1 and to any other anti-nominal antigen antibodies from another species and lack of reactivity with Ab_2 to other antibodies; (2) inhibition of the binding of Ab_1 to the specific antigen, the nominal antigen, and (3) the ability to elicit the synthesis of Ab_3 with anti-antigen specificity in animals without previous exposure to the antigen (Ertl and Bona, 1988).

The important role of anti-idiotypic antibodies in vivo has been shown in numerous experiments. The administration of anti-idiotypic antibodies was found to elicit different effects: either suppression or enhancement of the responses to the specific idotype(Hart, 1972, Kennedy, 1983). In autoimmunity, it certainly plays an important role. The pathology associated with many autoimmune diseases is most likely due to (at least in part), a direct idotype-anti-idotype interaction of the autoimmune antibodies with anti-idiotypic antibodies. Idiotypic specificity in a specific antibody were first characterized, by demonstrating that specific hapten binding could inhibit idotype recognition. The first experimental support for the validity of the internal image was presented by Sege and Peterson in 1978 by using anti-idotype as a probe to identify cell surface receptors.

The best information for the exact molecular basis for the mimicking presently is obtained from the X-ray crystallography of the idotype-anti-idotype complex. The basis of molecular mimicry of the antibodies can be either local sequence homology to the original protein as in a reovirus system or, in most cases, identical conformations from entirely different amino acid sequences as in the hemoglobin-myoglobin family of proteins. X-ray crystallography and sequence data in the later studies showed that identical, functional conformations can be assumed by proteins that differ by as many as 137 of 141 amino acids. The studies of the crystal structure of idiope-anti-idiope complex in the anti-

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lysozyme antibody and the anti-idiotope have demonstrated that a private idiotope consists of 13 amino-acid residues, most from the complementarily-determining regions, but including three residues from the third framework region of its VL domain. Seven of these residues are common with the paratope of anti-lysozyme antibody, indicating a significant overlap between idiotope and antigen-combining site. Idiotype has been a unique tool in characterization and manipulation of the immune response since it was found and realized: as a clonal marker to follow B cell development, somatic mutation and fate of clones of B cells. They have been used as a phenotypic marker for germ-line V genes. Anti-idiotypic antibodies which bear the internal image of external pathogens such as virus, bacteria or parasites have been used as surrogate antigens for vaccine and are being used in treating B cell lymphoma and autoimmune disease such as encephalomyelitis. In addition, it has been shown that anti-idiotypic antibodies can induce T-cell responses in which either cytotoxic T-cells or T-helper cells are produced which recognizes the original antigen.

The provision of a protective vaccine against chlamydial infections of the eye, genital tract, lung or heart would have worldwide public health benefit. The requirements for a successful vaccine candidate against *C. trachomatis* must include: (a) immunogenicity after presentation in an clinically safe carrier, (b) induction of neutralizing anti-chlamydial antibody, (c) reduction of clinical and histopathologic disease, and (d) absence of chlamydia-specific delayed hypersensitivity. Several chlamydial antigens have been shown to be immunogenic in patients and animal models. The major outer membrane protein (MOMP) has been the favored candidate antigen because of its immunogenicity, and the demonstration of neutralizing anti-MOMP monoclonal antibodies. However, various formulations of MOMP ranging from membrane extracts to fusion proteins containing MOMP subunits have been variably immunogenic, but none have protected against disease in the monkey model of trachoma.

Prior to the present invention, production of neutralizing antibodies by

using anti-idiotypic idotype antibody to mimic carbohydrate antigen have been produced in a bacteria system, Schriver et al, J. of Immunol; 144: 1023, 1990.

Accordingly, it would be desirable to provide a means for preparing a genus specific oral vaccine capable of providing immunization from chlamydial infection. It would also be desirable to provide a means for producing such an oral vaccine in quantity and to provide a process for increasing the effectiveness of the vaccine.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a binding curve of guinea pig antisera to monoclonal GLXA-Ab₁.

Figure 2 is a binding curve of guinea pig anti-monoclonal GLXA-Ab₁ IgG antisera to normal mouse IgG before and after immunosorption.

Figure 3 is a curve showing the inhibition of the binding of monoclonal GLXA-Ab₁ to GLXA by absorbed guinea pig anti-idiotypic antisera.

Figure 4 is a curve showing fractionation of guinea pig anti-idiotypic IgG.

Figure 5 is a curve showing the inhibition of the binding of monoclonal GLXA-AB₁ to GLXA by guinea pig anti-idiotypic isotypes.

Figure 6 is a curve showing the binding of rabbit anti-anti-idiotypic antibody to guinea pig anti-idiotypic IgG.

Figure 7 is a curve showing the binding of monoclonal GLXA-Ab₁ to GLXA by rabbit GLXA-Ab₃.

Figure 8 is a curve showing the detection of chlamydial specific ribosomal RNA from primates.

Figure 9 is a curve showing the effect of GLXA-Ab₃ IgG on ocular infection by clinical disease score.

Figure 10 is a curve showing the inhibition of bending of monoclonal GLXA Ab₁ to GLXA by a hybridoma clone.

Figure 11 is a curve showing the direct binding of chlamydia patient antiserum to monoclonal GLXA-M Ab₂.

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Figure 12 is a curve showing that rabbit anti-chlamydia antiserum recognizes monoclonal GLXA-M Ab₂.

Figure 13 is a curve showing the protection of mice from chlamydial infection by immunization with monoclonal GLXA-M Ab₂.

Figure 14 is a protection curve by monoclonal GLXA-M Ab₂ IgG after a high dose of ocular infections.

Figure 15 is a curve showing the effect of alum on the protection of mouse chlamydial infection.

Figures 16A and B show curves of the time course of ocular infectivity after immunization with monoclonal GLXA-Ab₂.

Figure 17 shows a typical timecourse of ocular infection in mice.

Figure 18 illustrates protection with anti-idiotypic antibodies against infection in mice in accordance with this invention.

SUMMARY OF THE INVENTION

The present invention is based upon the discovery that a vaccine, including an oral vaccine can be produced with anti-idiotypic antibody (hereinafter GLXA-Ab₂) which is capable of producing in an animal an anti-anti-idiotypic (hereinafter GLXA-Ab₃) antibody which recognizes GLXA, which is capable of immunizing an animal against chlamydia and is capable of neutralizing chlamydia infection in an animal. In addition, the useful anti-idiotypic antibody for producing the vaccine, including an oral vaccine can be either a polyclonal antibody or a monoclonal antibody. In addition, it has been discovered that these activities of the GLXA- Ab₂ surprisingly are obtained despite the fact that its predecessor, the idiotype antibody (hereinafter GLXA-Ab₁) from which Ab₂ is obtained does not have any significant activity in either immunizing or neutralizing against chlamydia infection.

By using the GLXA-Ab₂ to mimic carbohydrate of the glycolipid antigen, the present invention offers an alternative strategy for the conversion of a thymus-independent antigen into a thymus-dependent immunogen because

idiotype vaccines are proteins. The availability of GLXA-Ab₂ which mimics the chlamydial antigen, GLXA, in accordance with this invention, is of importance: (1) in the elucidation of the immunological mechanisms associated with the carbohydrate epitope present on GLXA (2) its function in chlamydial infection such as the attachment and phagocytosis of the host cells and (3) it provides an essential tool to identify a GLXA specific receptor of the host cells. It provides a means for producing a chlamydial vaccine.

Monoclonal GLXA-Ab₂ is an immunogen (i.e., capable of eliciting an immune response) and binding specifically with the products of that response whether they be antibodies and/or cell surface receptors of B cells or T cells. The rapid response to monoclonal GLXA-Ab₂ and the memory demonstrated in the re-challenge experiment discussed below in Example I and Figs 16A and 16B indicates that a T cell response has been stimulated. This means that the T helper cell is responding to an antigenic determinant (epitope) which differs from the GLXA epitope and is involved in the stimulation of B cells which produce an antibody receptor for GLXA-Ab₃ that binds GLXA. In addition, the T helper cell may be of the types which produces cytokines that have been shown to be involved in protective chlamydia infections. Two of these are gamma-IFN (gamma interferon) and TNF (tumor necrosis factor).

In accordance with this invention, there is provided: (1) production of polyclonal and monoclonal anti-idiotypic antibodies

selected for the internal image of the antigenic epitope on GLXA (2) characterization of the anti-idiotypic antibodies and (3) a vaccine including an oral vaccine which provides immunization, neutralization and protection of chlamydial infection both in vitro and in vivo and (4) a method for detecting the presence of chlamydia in a biological sample.

Particular mention is made of an oral vaccine for administration to an animal in an amount sufficient to effect immunization against chlamydia which comprises microspheres having a solid matrix formed of a pharmacologically acceptable polymer containing a biologically active composition, wherein said composition is selected from the group consisting of a monoclonal anti-idiotypic antibody, a Fab fragment of said monoclonal anti-idiotypic antibody, a (Fab)₂ fragment of said monoclonal anti-idiotypic antibody, and mixtures thereof, wherein said monoclonal anti-idiotypic antibody, said Fab fragment and said (Fab)₂ fragment induces in an animal anti-anti-idiotypic antibody which binds an epitope on genus specific chlamydia glycolipid exoantigen (GLXA).

Mention is also made of use of a biologically active composition which comprises microspheres having a solid matrix formed of a pharmacologically acceptable polymer, wherein said composition is selected from the group consisting of a monoclonal anti-idiotypic antibody, a Fab fragment of said monoclonal anti-idiotypic antibody, a (Fab)₂ fragment of said monoclonal anti-idiotypic antibody, and mixtures thereof, and wherein said

monoclonal anti-idiotypic antibody, said Fab fragment and said (Fab)₂ fragment induces in an animal anti-anti-idiotypic antibody which binds an epitope on genus specific chlamydia glycolipid exoantigen (GLXA) in an amount sufficient to effect immunization against chlamydia infection, for oral immunization of an animal against chlamydia infection.

DESCRIPTION OF SPECIFIC EMBODIMENTS

In accordance with this invention to produce a vaccine including an oral vaccine active against chlamydia infection, an antibody to GLXA is produced in a

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first step by any conventional method.

The GLXA is obtained and purified by any presently available method. Thus GLXA can be isolated on a octylsepharose column and eluted with alcohol or isolate on DEAE Sepharose and eluted in low pH aqueous solution or isolated on a polyacrylamide bead column and eluted with low pH aqueous solution of KSCN. (5M). In a preferred method GLXA is obtained and purified from supernatant of infected cell cultures by presently available methods such as by exclusion chromatography over a Sepharose 66B-Cl column in 0.075 M phosphate, 0.154 M NaCl, pH 7.2. The GLXA appears in the front fraction of the column and is detected by a chemiluminescence assay using an acridinium ester conjugated monoclonal GLXA-Ab₁. The fractions containing the GLXA are usually contaminated by nucleic acids, but not protein. The GLXA fractions are pooled, concentrated and treated with Rnase and DNase at pH 8.0 for about 2 hours at 37°C. The mixture is rechromatographed over the same column and is now pure. It can be stored at 4°C (with or without preservative).

The antibody can be polyclonal or monoclonal. In the production of monoclonal antibody, an idotypic antibody GLXA-Ab₁ is provided by immunizing an animal, usually a mouse, with GLXA or the chlamydia bacteria as the antigen. Immune spleen cells of the animal then are identified, isolated and fused with lymphoma or myeloma cells by being contacted with a fusing agent such as polyethylene glycol such as by the procedure of Kohler & Milstein, Nature 256: 459, 1975. The fused cells then are incubated in a selective medium such as HAT medium which precludes the growth of unfused malignant cells. The hybridoma cells are cloned by limiting dilution and supernatants are assayed for secreted monoclonal antibody of desired specificity. A suitable hybridoma for producing GLXA-Ab₁ is deposited in the American Type Culture Collection on March 12, 1993 and identified as ATCC H.B. 11300. Monoclonal antibodies also can be obtained by ascitic growth of hybridomas in vivo. Alternatively, the

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lymphocyte cells can be immortalized by exposure to Epstein-Barr virus. The idiotype antibody, GLXA-Ab₁, is useful in producing GLXA-Ab₂ which, surprisingly is active in immunizing against or neutralizing a chlamydia infection. In addition, GLXA-Ab₂ is not species specific but is genus specific in that its immunization and neutralization activity is useful in many animal species. The monoclonal GLXA-Ab₂ also can be produced by the process utilizing hybridomas set forth above for GLXA-Ab₁ but by utilizing GLXA-Ab₁ as the antigen. A suitable hybridoma for producing GLXA-Ab₂ is deposited in the American Type Culture Collection on March 12, 1993 and identified as ATCC H.B. 11301. GLXA-Ab₂ also can be produced as a polyclonal antibody and, as a polyclonal antibody, it is active for immunization against or neutralization of infection by chlamydia. Polyclonal antibodies are made in any conventional manner wherein a first animal is immunized with GLXA as the antigen and polyclonal GLXA-Ab₁, is isolated from the animal's serum. The polyclonal GLXA-Ab₁ or monoclonal GLXA-Ab₁ then is injected into a second animal of a different species from the first animal and polyclonal GLXA-Ab₂ then is recovered from the second animal's serum.

Monoclonal GLXA-Ab₂ or polyclonal GLXA-Ab₂ are both anti-idiotypic antibodies which mimic an antigen comprising a genus specific chlamydial glycolipid exoantigen (GLXA). Both forms of GLXA-Ab₂ are useful as a vaccine including an oral vaccine for immunizing an animal against genus specific chlamydial infection. In addition, both forms of GLXA-Ab₂ are useful for administration to an animal infected with chlamydia in order to neutralize genus specific chlamydia infection.

Also, in accordance with this invention, Fab or (Fab)₂ fragments vaccines suitable for immunizing against or neutralizing chlamydial infection can be prepared from the Fab or (Fab)₂ fragments. The Fab fragments can be prepared from GLXA-Ab₂ by any conventional means such as by exposing the GLXA-Ab₂ to papain or by exposure to trypsin or chymotrypsin followed by reduction and alkylation.

The vaccine of this invention including the oral vaccine, is prepared in a

form which permits it to pass through the stomach of a patient without degradation of the vaccine while permitting the vaccine to be taken up by the M cells in the Peyer's patches of the patient. The vaccine comprises microspheres having a diameter between about 1 and about 100 microns, preferably between about 5 and 20 microns. The microspheres are formed from a physiologically acceptable polymer degradable in a patient including synthetic polymers such as poly(lactic acid), polylactide, poly(glycolic acid), poly(glycolide), poly(lactide-co-glycolide), poly(caprolactone), polyanhydride, poly(ortho esters), polyphosphates, polyphosphonates, polyphosphazenes or the like or naturally occurring polymers such as gelatin, collagen, albumin, or other proteins, polysaccharides or the like.

In accordance with this invention an organic solution of the physiologically acceptable polymer is formed at a polymer concentration of between about 1 and about 50 w/v %, preferably between about 20 and 40 w/v % in a physiologically acceptable volatile solvent such as methylene chloride, acetone, acrylonitrile or the like.

A second solution is formed from water either in the presence or absence of a buffer for the polyclonal or monoclonal GLXA Ab₂ or the Fab or (Fab)₂ fragment of the polyclonal or monoclonal antibody GLXA-Ab₂. Any suitable buffer can be utilized so long as it does not degrade either the GLXA-Ab₂ or (Fab)₂ fragment thereof and does not degrade the polymer forming the microsphere shell. Representative suitable buffers include, phosphate, borate, bicarbonate or the like. The ionic strength of the buffer solution should be less than about 10 mM so that excessive salt is not present during solvent evaporation and lyophilization. If excess buffer is present, both the GLXA-Ab₂ and the microencapsulation process can be diversely affected. Thus, if present, excess buffer is removed from the antibody solution by any conventional means such as by dialysis.

The organic solution of polymer and the aqueous solution of antibody then is mixed such as by stirring to form a water in oil emulsion wherein the antibody is solubilized in the aqueous phase and the polymer is solubilized in the organic

phase.

In order to stabilize the emulsion and to form microspheres of a matrix of the polymer which contains the antibody and/or the FAB or (Fab)₂ fragment thereof, the emulsion is admixed with water containing a surfactant which stabilizes the emulsion.

A suitable surfactant is polyvinyl alcohol (PVA) such as those having a molecular weight between about 10,000 and about 35,000, e.g., about 25,000.

The resultant emulsion then is admixed such as by pouring into a second aqueous solution of the surfactant such as PVA at a suitable surfactant concentration such as between about 0.1 and 0.8 weight %, preferably between about 0.3 and 0.5 weight %. The resultant mixture is stirred for a suitable time such as 2 to 10 hours in order to permit the microspheres to form. The microspheres then are recovered such as by centrifugation and lyophilization. The recovered microspheres containing the GLXA-Ab₂ antibody or the Fab or (Fab)₂ fragment thereof in the polymer matrix then is in a form suitable as a vaccine including an oral vaccine such as by being mixed with water.

The following examples illustrate the present invention and are not intended to limit the same.

Example 1

Throughout out this study, two serovars of C. trachomatis were used. Serovar B (Strain Har 36) organisms originally isolated from conjunctival scraping of a child, were grown at 37° C on McCoy cell monolayer with Eagle's Minimum Essential Medium with Hank's balanced salt solution (HMEM, Whittaker, MD) and stored at -70° C in the same medium with 10% of dimethyl sulfoxide (Sigma Chemical Co., MO). Serovar C (TW-3) was grown in mass tissue culture in McCoy cells. The elementary bodies were purified by centrifugation through renograffin and resuspended in phosphate-buffered saline and stored at -70° C.

Chlamydia free Hartley strain inbred guinea pigs were originally obtained from Jackson Laboratory (Bar Harbor, ME), bred and maintained in an animal

facility. One year old females were used in this study.

Female New Zealand rabbits free of Pasteurella were obtained from Mill Brock Farm (Hadly, MA). The rabbits were about 6-8 pounds when received and used within one week. BALB/c ByJ (H-2d) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Young adult cynomolgus monkeys (Macacca fasciuiareis) were obtained from Charles River Primates, Inc. (Port Washington, NY) which were free of clinical ocular diseases. All the primates had been used for a previous ocular infection with C. trachomatis serovar C elementary bodies, some primates had been previously immunized with a chlamydial protein. The primates challenged at this study, were free of the disease and were not immune. They were randominized prior to the initiation of the experiment. All procedures were performed under anesthesia.

For the production of polyclonal anti-idiotypic antibodies, each of six guinea pigs was immunized subcutaneously with 150 ug of monoclonal GLXA-Ab₁ (89MS30) IgG in 1 ml of H₂O plus 1 ml Maalox Plus* suspension (William H. Roger, Inc., PA). Three weeks later the guinea pigs were boosted with 100 ug of monoclonal GLXA-Ab₁ in 1 ml of Maalox* (aluminum hydroxide, alum) and H₂O, and were boosted on a monthly basis.

For the production of anti-anti-idiotypic antibodies, three rabbits were immunized with absorbed anti-idiotypic guinea pig isotype IgGI, 300 ug in 1 ml of alum and 1 ml of H₂O intradermally on multiple sites and boosted three weeks later

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using the identical protocol. Subsequently, rabbits were boosted at monthly intervals. For the production of monoclonal anti-idiotypic antibody, GLXA-Ab₂ (91 MS441), fifty ug of KLH conjugated monoclonal GLXA-Ab₁ IgG was injected intraperitoneally into five 9-week old female BALB/cByJ mice in an equal amount of complete Freund's adjuvant (Sigma, MO). The same rabbit was used in the subsequent booster at day 14 and later on a weekly basis for five weeks in the presence of incomplete Freund adjuvant. A final booster injection was given 3 days before the removal of the spleen. For the protection of chlamydial infection experiment, two groups of BALB/c ByJ mice (6 to 20 in each group) were immunized with 50 ug of monoclonal GLXA-Ab₂ IgG or 50 ug of normal mouse IgG per mouse subcutaneously with or without Maalox and boosted on weekly basis for three weeks.

Monoclonal GLXA-Ab₁ IgG was isolated by protein A affinity chromatography. The ascites which contains monoclonal GLXA-Ab₁ IgG was first passed through glass wool to remove lipid and centrifuged 2000 rpm for 10 minutes to remove precipitates. The affinity column (1.5 x 9 cm) was packed with 2 ml Protein A Sepharose CL4B* (Zymed, CA) was first equilibrated in a binding buffer (1.5M glycine, 3M NaCl, pH 8.9) Jackson Immuno Research Laboratories, PA). Two ml ascites diluted with equal amount of binding buffer was loaded onto the column at 0.6-0.8 ml/minute. The column was stopped for about 30 minutes to allow binding and then rinsed with about three bed volume of binding buffer. The

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bound IgG was eluted with 0.1 M citric acid, pH 3, into glass tubes which contained 50 ul 1 M TBS, pH 8.0 to equilibrate the pH. IgG was detected by an absorption at 280 nm. The absorption larger than 0.1 was pooled and dialyzed against 0.075 M PBA, pH 7.2 overnight. The purity of the IgG isolated was confirmed by SDS PAGE on a Phastsystem* (Pharmacia, NJ).

Direct enzyme-linked immunosorbant assay

The specific response of guinea pig immunized with monoclonal Ab₁ IgG per well in coating buffer (0.015 M NaHCO₃, 0.3M glycine, 0.02% NaNO₃ with 0.06M polyethylene glycol, PEG), pH 9.6. The plate was stored at 4°C overnight. Each plate was rinsed three times with 0.05% Tween 20* in 0.075M PBS and blocked with 1% BSA/PBS for 2 hours at room temperature. Again, the plate was rinsed three times. The pre-immune sera or antisera from guinea pigs were serially diluted with 0.075M PBS and 100 ul of each was added to each well in duplicate. The mixture was incubated at room temperature for 1 hour and rinsed. Goat anti-guinea pig IgG (H & L) horseradish peroxidase conjugate (Jackson Immuno Research Labs, Inc. PA) 1:1000 was added, incubated for one hour and rinsed. IMB substrate (Kirkegaard and Perry Laboratories, MD) was added after rinsing. The absorbance at 405 nm was determined using a Vmax microwell reader (Molecular Devices Corp., CA). The antisera from rabbits immunized with guinea pig IgGI was assayed in the similar manner. Each plate was coated with guinea pig IgGI and the second antibody was goat

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anti-rabbits (H & L) horseradish peroxidase conjugate (Jackson Immuno Research Labs. Inc., PA).

Preparation of Affinity Chromatography Column

Normal mouse IgG (Jackson Immuno Research Labs Inc., PA) was conjugated to Affi-Gel IO* (Bio-Rad Laboratories, CA) as indicated by the manufacturer. Briefly, 5 ml Affi-Gel IO slurry was transferred to a glass fitted funnel connected to an aspirator and washed with three bed volumes of isopropyl alcohol followed by three beds of ice cold deionized water. Normal mouse IgG (5 mg/ml), 10 ml was mixed with Affi-Gel IO in a vial. The coupling was done at 4°C overnight with gentle end-to-end agitation. A column (0.9 x 5ml) was packed with the coupled gel and rinsed with 0.075M PBS, pH 7.2. The UV absorbance at 280 nm of the effluent was monitored. The highest absorbance of this portion was used to test for protein content by the Bradford Assay (Bio-Rad Laboratories, CA) to evaluate the conjugation.

Absorption of guinea pig antisera with normal mouse IgG

A pool of one guinea pig antisera 3, 4 and 5 weeks after the immunization was absorbed by affinity chromatography on a normal mouse IgG-agarose column. The column was prepared as above. The antisera, about one void volume, was loaded onto the column and incubated for 30 minutes at 4°C and eluted with 0.075M PBS, pH 7.2. The antiserum was absorbed a second time

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with a freshly prepared column.

Separation of isotypes of guinea pig IgG

Five ml of guinea pig antisera which had been absorbed by normal mouse IgG, was loaded onto a protein A conjugated sepharose column, and rinsed with 0.02M phosphate-citrate buffer, pH 7.3. Guinea pig immunoglobulin subclasses IgG1 and IgG2 were eluted separately using a step pH gradient of 4.9 until no protein was detected in the effluent. The column was then eluted with a low pH gradient, 4.3. After dialysis against 0.02 M phosphate-citrate buffer, pH 7.3, each isotype was subjected to a second round of subclass separation.

C. trachomatis serovar B (Har. 36) elementary bodies were propagated in a McCoy cell monolayer in a 2 liter roller bottle (Corning, PA) in HMEM with added L-glutamine (final concentration 0.5 mM), NaHCO_3 (final concentration 0.4 mM) and 10 % of fetal bovine sera (FBS). Briefly, the medium was removed from the bottle after confluence of the monolayer. Then, 0.5-3.0 ml of EBs suspension (depending on the density of the organisms) in 40 ml of cycloheximide overlay medium (COM) (Whittacker, MD) with added L-glutamine and FBS was inoculated and the bottle was rolled at 3 rpm at 35° C. Two hours later, 200 ml of COM was added and rolling continued for 48 to 72 hours. The supernatant was obtained after centrifugation to remove organisms.

Purification of glycolipid exoantigen, GLXA

GLXA was purified by two steps from the supernatant. First, the supernatant was passed through an octyl-sepharose CL4B column and eluted with 95% ethanol (Stuart and MacDonald, Current Microbiology, 11:123, 1984). The antigen in ethanol (antigen content equivalent to 50 ml heavily infected tissue culture supernatant) was concentrated to approximately 50 ul and resuspended in 0.1M phosphate buffer, pH 7.5. to 1 ml. Second, antigen thus prepared was

further purified by affinity chromatography. The affinity column was prepared by conjugating monoclonal GLXA-Ab₁ IgG to Affi-Gel IO (Bio-Rad Laboratories, CA) as instructed by the manufacturer. The sample was centrifuged and loaded on to the affinity column and incubated for 30 minutes at 4°C. The column was rinsed with 0.1 M phosphate buffer until the eluant became clear. GLXA was eluted in approximate 5M of potassium thiocyanate (KSCN, Mallinckrodt Inc., KY) for about 15-20 ml and dialysed against 0.075M PBS overnight.

GLXA used in the immuno-dot blot assay of binding of GLXA and monoclonal GLXA-Ab₂ by monoclonal GLXA-Ab₃ described below was isolated by a size exclusion method. Sepharose 6BLC column (2x100 cm) was equilibrated with 0.075M PBS. The supernatant from C. trachomatis serovar F cell culture was first centrifuged 5000 rpm to remove cell debris and approximately 20 ml was loaded onto the column. The flow rate was 0.5 ml/minute. GLXA in the eluant was detected with Magic Lite Analyzer* (Ciba Corning Diagnostic Corp., MA). The protocol is as described below.

Chemiluminometric immunoassay (Inhibition Assay)

Inhibition of the binding of monoclonal GLXA-Ab₁ IgG to GLXA by guinea pig GLXA-Ab₂ or rabbit GLXA-Ab₃ (antisera, IgG, IgG isotypes) was detected by chemiluminometric immunoassay with a Magic Lite Analyzer (Ciba Corning Diagnostic Corp., MA). The protocol for the inhibition was as follows: Affinity purified GLXA was diluted 1:5 with reagent A (Ciba Corning), mixed and

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aliquoted into plastic tubes (200 ul/tube, Sarstedt, NJ). A neutralizing solution Reagent B (Ciba Corning), 100 ul was added to each tube and mixed well. Serial dilutions of guinea pig GLXA-Ab₂ or rabbit GLXA-Ab₃ IgG, 50 ul were added to each of the above tubes and incubated at room temperature. One hour after the incubation, 100 ul acridium ester-labeled (AE-labeled) monoclonal GLXA-Ab₁ IgG in 1% BSA/PBS (between 220,000 to 240,000 relative light units, RLU) was added into the mixture and incubated for one hour. Then, polyclonal anti-chlamydial antibodies which were covalently bound to paramagnetic particles (500 ul) was added to each tube and incubated for another hour. The bound immune complex (paramagnetic particles-GLXA-AE labeled monoclonal GLXA-Ab₁ IgG) is separated from the unbound by subjecting the mixture to a magnetic field. The particles were rinsed 3 times. Bound AE-labeled antibody was measured by the Magic Lite Analyzer and RLU were recorded. The percentage of inhibition of binding was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{RLU control} - \text{RLU test}}{\text{RLU control}} \times 100$$

where control represents PBS and test represents either pre-immune or antisera (or IgG) added respectively.

Immuno-dot blot Assay

A Bio-rad dot blot apparatus was used for this assay.

Polyvinylidene fluoride (PVDF) membrane, 0.45 um, Immobilon-NC* (Millipore, MA) was coated with the affinity-purified GLXA or chlamydial rLPS for 18 hours at 4°C. The membrane was then washed with 0.05% Tween 20 in 0.075M PBS buffer, pH 7.2 and blocked for 2 hours at room temperature with 3% BSA/PBS. After rinsing, serial dilutions of rabbit GLXA-Ab₃ IgG (90MS699) (isolated by protein A affinity chromatography, method see above) or monoclonal Ab₁ IgG (89MS30) were added (100 ul per well), incubated at room temperature for 2 hours, and washed to remove unbound IgG. The PVDF sheet was removed from the apparatus and blocked with 3 % BSA/PBS for two hours with gentle agitation, then probed with 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG (H and L) or rabbit anti-mouse IgG (H and L) (Jackson Immuno Research Labs., PA) for 1 hour at room temperature. Unbound conjugated antibody was removed by washing in a petri dish for 4 times, 5 minutes with agitation. The binding was detected by 4 CN (4-chloro-I-naphthol) membrane peroxidase substrate system

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(Kirkegaard & Perry Laboratories Inc., MD). Assays were carried out in duplicate.

Biotinylation of IgG

Monoclonal GLXA-Ab₁ or rabbit GLXA-Ab₃ IgGs were labeled with biotin. Briefly, the GLXA-IgG was first dialysed against 0.1 M sodium borate, pH 8.8 for 4 hours at 4° C. Then, 200 ug of biotinamidocaproate N-hydroxysuccinimide ester (Sigma Chemical Co., MO) per mg of IgG was added, and incubated at room temperature for 4 hours. Finally, 20 ul of 1 M NH₄Cl per 250 ul of ester was used and incubated for 10 minutes at room temperature to stop the reaction. Labeled IgGs were dialysed against 0.075M PBS for 3 days at 4° C.

Immunofluorescence staining of infected McCoy cells

Confluent McCoy cell monolayers were grown on the cover slips in a 24 well plate (Falcon, NJ) for 24 hours. The medium was taken out from each well before 200 ul of C. trachomatis serovar B elementary bodies (Har 36) or 200 ul of HMEM alone were applied to each well. The inclusions forming units (IFU) was approximately 1000/200 ul. Immediately after the application, 200 ul of cycloheximide overlay medium (COM) with L-glutamine and FBS was added to each well. The mixture was incubated at 37° C for two hours and the medium was replaced by 1 ml COM per well. The plates were then cultured for 48 hours at 37° C in a humidified incubator with 5% CO₂.

After the incubation, cell cover slips were washed with 0.075M PBS twice, 5 minutes each. The coverslips were then blotted with tissue carefully to remove buffer on the cover slips. Then 400 ul of 50 ug/ml biotin-labeled monoclonal GLXA-Ab₁ or 400 ul of 1.20 mg/ml of biotin-labeled GLXA-Ab₃ was added to the cover slips and incubated at 37° C for 1 hour. The cover slips were rinsed twice with PBS (five minutes each rinse). Four hundred ul of fluorescein-streptavidin

1:50 in 1% of BSA/PBS was added and incubated for 1 hour. The unbounded fluorescein-streptavidin was rinsed out as above. Fluorescence was detected by photograph with an Olympus 25, 35mm camera (ASA 400 TMAX black and white Kodak film) mounted on a Zeiss A.7082 Oberkichen microscope, illuminated with a 12v/100w halogen lamp.

Neutralization of Chlamydia by GLXA-Ab₃ (9OMS699) in vivo

Inoculums and specimens

The rabbit pre-immune and GLXA-Ab₃ IgG, normal mouse and monoclonal GLXA-Ab₁ IgG were filter sterilized. The IgGs (0.2 mg/ml) were mixed (1:1) respectively with C. trachomatis serovar C (TW-3) elementary bodies (2000 inclusion-forming units/20ul). The mixtures and non-treated EBs (as a control) were incubated at 37° C for 45 minutes, with gentle shaking every 15 minutes.

After the incubation, approximately 2 ug IgG plus 1000 IFU in 20 ul was inoculated onto inferior fornix of each eye of eight monkeys, four with GLXA-Ab₃ IgG plus EBs, two with pre-immune IgG plus EBs and two with EBs only. At the same time, 100 ul of each type of mixture was used to infect 10 wells of 2-day-old McCoy cell monolayer coverslip in a 48 well tissue culture plate for determination of the infectivity of the inoculum. Culture method are described below.

Conjunctival swabs were taken by sweeping the interior tarsus and fornix, the lateral fornix, the superior tarsus and fornix, and the medial fornix on the day prior to inoculation, and on days 2, 6, 9, 13, and 20 after the inoculation. The swabs were immediately immersed in 2 ml collection medium and disrupted by vortexing for 2 minutes in the collection medium.

Determination of chlamydial infectivity by cell culture

Conjunctival swabs were first disrupted by vortexing for two minutes in the collection medium to collect EBs from the swabs. Then 200 ul of this collection medium was inoculated onto McCoy cell monolayer cover slips. The cells in a 48 well tissue culture plate were grown for two days, 5 wells for each sample. For an in vitro neutralization experiment, 100 ul of the mixture was inoculated onto the monolayer cover slips of 10 wells for each sample. The inoculated plates were then centrifuged at 1,000 x g for 1 hour at room temperature. The plates were incubated at 37°C for 2 hours and the medium was replaced by 1 ml of 10 % FBS in COM (Whittaker, MD). The plates were cultured at 37°C in a humidified incubator with 5% CO₂ for 48 hours. The culture medium was aspirated from each well after the incubation. The monolayer coverslips were washed once with 0.075M PBS, fixed with ethanol for 5 minutes and then rinsed with H₂O twice. The fluorescein-labeled rabbit anti-chlamydial monoclonal antibody with Evans blue counterstain (Syvo, Co., CA.) 25 ul per well was added and incubated for 30 minutes at 37°C. After the incubation, the wells were rinsed with H₂O twice and a cover slip was mounted to each well with mounting fluid. The inclusion bodies were counted by inverting the plate on a fluorescence microscope.

Direct Florescent antibody-stained cytology (DFA)

The conjunctival swab (scraping) from each eye of infected

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primate was pressed on alcohol-precleaned glass slide. The slides were air dried and fixed with cold acetone for 5 minutes. Slides were then dried and 30 ul of fluorescein-labeled monoclonal antibody reagent with Evans blue counterstain (Micro Trak* Chlamydia Direct Reagent, Syvo Co. CA) were overlaid. The incubation was carried out in a covered, moist chamber. After 15 minutes, slides were turned on edge to remove excess stain and rinsed with deionized water for 10 seconds. Slides were allowed to completely air dry. A cover slip was placed on each slide

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with mounting medium (Syva Co., CA) and sealed with fingernail polish. Slides were examined under a fluorescence microscope at 500x and 1250x. The infectious titer was scored on a semi-quantitative scale 0-4+: 0 is negative 0.5 is negative on first passage and positive to any degree of second passage, 1 is for 1 to 9 inclusions per well on first passage and so on.

Clinical examination and scoring

The clinical response of each eye was graded as ten clinical signs (Taylor et al, Invest. Ophthalmol. Vis. Sci. 29:847, 1988): the follicular response in the lumbar, limbal, superior tarsal, superior fornix, and inferior fornix portion of the conjunctiva hyperemia or injection of the bulbar, superior tarsal, superior fornix, and inferior fornix conjunctiva and ocular discharge. The clinical response was graded on a scale of 0 to 3 for each of 10 signs of conjunctival inflammation to obtain a total inflammatory score for each monkey. The examiner was unaware about the allocation of the monkeys. The means of the scores were used to describe the response of a group of monkeys.

Analysis of Conjunctival Swabs via RNA-directed Hybridization

Total RNA was prepared from conjunctival swab samples taken from GLXA-Ab₃ or normal rabbit IgG treated monkeys on the day before the challenge and on day 2, 6, 9, 12, and 20 after the challenge. The swab samples were first homogenized in phenol at 65% in the buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 % SDS. RNA was precipitated and redissolved in the same buffer. This preparation of RNA was extracted several times with phenol:chloroform (3:1), resuspended and treated extensively with DNase I. The quality of RNA preparation was monitored by ethidium bromide-UV visualization after separation on formaldehyde-agarose gels.

The probe used was a DNA fragment containing the 16S and 23S ribosomal RNA and flanking sequences which was excised from the chlamydial genomic plasmid clone pL2, 434Scl-IA (Cheema et al, The Amer. J. Med. Sci. 302:261-268, 1991). The DNA fragment was labelled by nick translation using ^{32}P dCTP, 800 Ci/m mole (Amersham Corp., IL). The specific activity for all restriction fragment probes was about 106 cpm/ug DNA. Slots on each blot included 1 ug of monkey or human-derived total RNA, 10 pg pure C. trachomatis or C serovar RNA (positive control), 3 ug yeast or rat RNA, buffer alone and 1 ug RNA from swabs of each monkey taken prior to infection (negative control). RNA was fixed to 0.22 um filter (Schleicher and Schuell Corp., NH). The hybridization results were visualized via autoradiography at -70° C using X-OMAT AR film (Kodak, New York).

Production and Characterization of Monoclonal Anti-idiotypic Antibodies (monoclonal GLXA-Ab₂)

Conjugation of keyhole limpet hemocyanin with monoclonal GLXA

Monoclonal Ab₁ (89MS30) IgG was isolated from a protein A chromatography column as set forth above. The keyhole limpet hemocyanin conjugation was carried out by using glutaraldehyde. Briefly, 1.5 mg of IgG was mixed with 0.05 mg KLH, (Sigma Chemical Co., MO) (approximately 1 molar of IgG per 50 amino acid of KLH) in equal volume of 0.2 % of glutaraldehyde in PBS, incubated at room temperature with gentle stirring. One hour later, 1 M of glycine was added to make a final concentration of 0.02M and incubated for another hour at room temperature. The conjugate was then dialysed against PBS overnight.

Anti-monoclonal Ab₁ hybridoma production

Four days after the last boost (see immunization), spleen cells were isolated from two mice which had the highest titer. Fusion was made with mouse myeloma cell line Sp2/0-Ag14 according to the techniques initially developed by Kohler and Milstein (Nature, 1975) and modified (Goldsby, A Practical Guide to Making Hybridomas In Nucleic Acid & Monoclonal Antibody Probes, Swaminathan & Prokask, Eds. Deller. N.Y. pg. 367, 1989). Feeder cell layer were from spleens of mice 8 weeks old.

Screening of Monoclonal anti-idiotypic antibody, 91MS441 (monoclonal GLXA Ab₂)

Anti-idiotypic antisera from immunized mice and supernatants from cloned wells were detected by a sandwich ELISA (Uytdehaag et al, J. Immunol, 134: 1225, 1985, Hiroshima et al, J. Immunol. 144: 224, 1990). Briefly, polystyrene Immulon II microtiter plates (Dynatech Laboratories Inc., VA) were coated with 100 ul of 1 ug monoclonal Ab₁ IgG in 0.1 M carbonate buffer, pH 8.9 overnight at 4° C. The unbound IgG was removed and the wells were blocked by 3% BSA/PBS for 1 hour at 37° C. After washing, serial diluted antisera or 100 ul of culture supernatant from wells with hybridoma cells was added. After 1 hour incubation at 37° C and washing, 1 ug of biotin conjugated monoclonal Ab₁ IgG was added to each well and the reactivity was detected by the addition of streptavidin-horseradish peroxidase (Jackson immuno Research Labs., PA). One hour later, TMB peroxidase substrate (Kirkegaard & Perry Laboratories inc., MD) was added. The absorbance was read at 405 nm in a microplate reader. As positive and negative controls, immune sera taken before the fusion (1:10 dilution) and medium alone were used.

Inhibition of binding Assay

The inhibition of the binding of monoclonal GLXA-Ab₁ to GLXA by mouse antiserum and the supernatant of the clones was determined by immuno-chemi-

luminometric assay as described above.

Generation of ascites

Ten BALB 3/cByJ mice, age not strictly required, were injected with Pristane* (2,6,10,14-tetramethylpentadecane, Sigma Chemical Co., MO), 1 ml per mouse intraperitoneally. Ten days later, the mice were injected with approximately 2×10^8 monoclonal GLXA-Ab₂ (91 MS441) producing hybridoma cells. The ascites was harvested by using a Vacutainer* 20 g blood collection needle (Becton Dickinson, NJ.) about 10 minutes and stored at -20°C until use.

Isotyping of anti-idiotypic IgG

The isotyping was carried out by ELISA. The supernatant from clone 91 MS441, (100 ul) was coated in each well and incubated at 4°C overnight. After rinsing and blocking with 3% BSA/PBS for 2 hours, 100 ul of rabbit antiserum specific to mouse subclass; IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, K chain or λ chain (Bio-Rad Laboratories, CA) was added to each well in duplicate. The plate was incubated at room temperature for one hour. The rabbit antiserum was detected by horseradish peroxidase conjugated goat anti-rabbit (H and L) and TMB substrate (Kirkegaard & Perry Laboratories Inc. MD.)

Purification of monoclonal GLXA-Ab₂ IgG was purified by affinity chromatography on a protein-G-sepharose column. Protein G-

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Sepharose 4B (Zymed, CA.) 5 ml was packed in a 0.5 x 9 cm column. The ascites (2 ml) was diluted 1:1 with 0.02 M phosphate buffer pH 7.3 and loaded on the column and washed with the same buffer until no protein was detected. The bound IgG was eluted with 0.1 M citric-glycine buffer, pH 2.6. The eluent was collected in 1 ml fraction which contained 50 ul of 1 M tris-saline buffer, pH 8.0 to balance the eluent. Fractions having UV

absorption above 0.1 were pooled and dialysed in PBS overnight at 4° C. The purified IgG was identified by SDS-PAGE for confirmation.

Immunoprecipitation of monoclonal GLXA-Ab₂ by anti-chlamydial antisera from other species

Polyclonal antibodies from a human patient diagnosed with a chlamydial infection and chlamydial EBs injected rabbits were tested for the recognition of monoclonal GLXA-Ab₂ by ELISA. The procedure was essentially the same as described above with the following exceptions. Briefly, the ELISA plate was coated with 1 ug of monoclonal GLXA-Ab₂ per well in 0.075M PBS without coating buffer overnight at 4° C. The wells were rinsed with 0.05% Tween 20 in 0.075M PBS and blocked with 3% BSA/PBS for 2 hours at room temperature. Serial dilution of the patient's serum (92MS273), control human serum (88MS356), rabbit antisera (88MS188) or control rabbit serum (92MS450) 100 ul was added to each well in duplicate. After 1 hour incubation at room temperature, the wells were washed 3 times and 100 ul of peroxidase conjugated goat anti-human or goat anti-rabbit IgG (Jackson ImmunoResearch Labs, MD) was added. TMB substrate was added after one hour incubation. The plate was read on a Vmax microplate reader (Molecular Devices Corp., CA).

Protection from Chlamydia Infection by Immunization of Monoclonal GLXA-Ab₂ in a Mouse Infection Model

Immuno-dot blot assay of binding of GLXA and GLXA Ab₃ raised by monoclonal GLXA-Ab₂

Immuno-dot blot assay was done by the same method as described above. The exception is that a PVDF sheet was coated with purified GLXA (100 ul) per lane in 0.075M PBS. Antisera taken from the mice which were immunized with monoclonal GLXA-Ab₂ IgG or normal mouse IgG were serially diluted with

3% BSA/PBS. The second antibody was horseradish peroxidase conjugated rabbit anti-mouse IgG (H and L). The photograph was taken by Kodak TMAX 100. The staining intensity of dot blot was scanned by a densitometer.

Inoculation and specimens

C. trachomatis serovar C (TW-3) elementary bodies 5000/20ul were inoculated onto each eye of the mice which were immunized with monoclonal Ab₂ or normal mouse IgG. On the day before the inoculation and on day 7, 10, 14, 21, 28 and 35 after the inoculation, conjunctiva were swabbed from each eye. The area included the inferior tarsus and fornix, the lateral fornix, the superior tarsus and fornix, and the medial fornix. The conjunctival swabs were immediately immersed in the collection medium and disrupted for two minutes by vortex and kept on ice until culturing.

Identification of Receptor on Host Cells by Monoclonal GLXA-Ab₂ IgG

FACS analysis of the specific binding of monoclonal GLXA-Ab₂ to HECEC cells

Human endometrial gland epithelial cells (HECEC) were grown in a 75 mm² flask at 37° C with 5% CO₂. When confluent, cells were scraped off the flask using a cell scraper (Baxter, IL) and centrifuged 200 x g for 5 minutes. The cells were rinsed once with 20 % FBS in Hanks buffer (Whittaker, MD) and passed through a 19 G syringe needle four times to obtain single cells. Serial dilutions of biotin labeled monoclonal GLXA-Ab₂ or biotin labeled normal mouse IgG in Hanks buffer, (100ul) were added to each vial which contained approximately 1.5 x 10⁶ HECEC cells and incubated on ice for 30 minutes. Each vial was rinsed twice with 0.02 % azide in Hanks buffer. Later, 100 ul of FITC conjugated streptavidin was added and incubated for 30 minutes on ice. After washing twice, the cells were kept in 400 ul of sheath buffer on ice. Cells plus FITC conjugated streptavidin and cells alone were used as background control.

Single color flow cytometry was performed immediately by FACS scan (Becton Dickinson).

Detection and Characterization of Polyclonal Anti-idiotypic Antibodies

Generation of anti-idiotypic antibodies against monoclonal GLXA-Ab₁ in guinea pigs

The immunogen which was used to produce the anti-idiotypic antibodies in guinea pigs was a monoclonal antibody identified as 89MS30 (monoclonal GLXA-Ab₁). It was originally produced by immunization of BALB/cByJ mice with chlamydial elementary bodies propagated in embryotic egg. Mice spleenocytes were fused with Sp2/0-A14 myeloma cells and the clone was screened. The clone (89MS30) reacted to all 15 serovars of C. trachomatis, C. pneumoniae, and C. psittaci 6BC and mouse meningopneumonitis by EIA, demonstrating recognition of a genus specific antigen. The IgG was isotyped as IgG2Bb by ELISA using rabbit anti-mouse antiserum (Bio-Rad Laboratories, CA). The monoclonal GLXA-Ab IgG was isolated from the ascites with rec-protein A sepharose 4B conjugate column (ZYMED, CA). Inbred guinea pigs (Hartley 13) were immunized and boosted with monoclonal GLXA-Ab₁ IgG, 150 ug each in the presence of Maalox, as an adjuvant. Pre-immune sera and antisera were obtained by heart puncture and centrifugation. Five immunized guinea pigs demonstrated strong immune responses against monoclonal Ab₁ IgG by ELISA. It was demonstrated that the anti-monoclonal GLXA-Ab IgG titer was more than 1 to 20,000 one week after the first boost by ELISA for anti-idiotypic. Pre-immune sera (□); antisera 3 weeks (⊗) after the immunization; 1 week (■) and 2 weeks after the boost (□). Each value represents the mean of the duplicated determinations. These guinea pig antisera kept increasing two weeks after the boost as shown in Figure 1.

Complete absorption of guinea pig antisera with normal mouse IgG.

In order to remove guinea pig anti-mouse antibodies that are directed against epitopes other than the idiotopes present on the hypervariable regions of

the monoclonal Ab₁ IgG molecules, the guinea pig antisera was absorbed with normal mouse IgG. The absorption was carried out by affinity chromatography. The column was made by conjugating normal mouse IgG to Affi-Gel 10. The conjugation was evaluated by detecting the amount of unbound protein at absorbance at 280 nm. The highest optical density from a fraction was 0.23, containing 0.18 mg of protein by the Bradford Assay (Bio-Rad Laboratories, CA). The total mouse IgG used in the conjugation was 50 mg., demonstrating that the conjugation was successful. The guinea pig antisera were loaded onto the column and eluted with 0.075M PBS, pH 7.2. this procedure was repeated using a newly prepared column. The antisera before and after the absorption was tested and compared with pre-immune sera for the reactivity to normal mouse IgG by ELISA. The concentration of each antiserum for the assay was equilibrated. Each well was coated with 1 ug of normal mouse IgG. Serial dilutions of pre-absorbed (•); pre-immuned (■) and absorbed guinea pig natisera (□) were added. PBS was background control (O). Horse radish peroxidase conjugated goat anti-guinea pig IgG (H&L) was used as the second antibody. Each value represents the mean of duplicate determinations. The result showed that sera after the absorption had the same reactivity against normal mouse IgG as pre-immune sera (Fig. 2). The unabsorbed antisera had 5 times more reactivity, indicating that all antibodies specific to epitopes other than idiotypes have been completely removed.

Inhibition of the binding of monoclonal GLXA-Ab₁ to GLXA by guinea pig antisera

If guinea pig antisera contain the specific anti-idiotypic antibody against monoclonal GLXA-Ab₁ IgG molecules, the direct effect is that it would inhibit the binding of antigen, GLXA, to the monoclonal antibody, monoclonal GLXA-Ab₁. In other words, the antisera bind to the complementary-determining region of monoclonal GLXA-Ab₁ IgG, thus preventing GLXA from binding to the active site. To test this, competition of

binding was performed by chemiluminometric immunoassay. Serial dilutions of guinea

pig pre-immune sera, unabsorbed antisera, or absorbed antisera were incubated with GLXA which was isolated by immunoaffinity chromatography. After one hour at room temperature, the mixtures were incubated with monoclonal GLXA-Ab₁ IgG conjugated with acridium ester for

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an additional hour. Solid phase paramagnetic particles were added and a RLU was determined. The inhibiting percentage is calculated as mentioned in Material and Method. Guinea pig anti-idiotypic antisera exhaustively absorbed with mouse IgG (■) inhibits the binding of mAb₁ to GLXA at approximately the same dilutions as unabsorbed guinea pig antisera (□) by chemiluminometric immunoassay. Pre-immune (○); mAb₁ (•); and 2X PBS (◆) were included as controls. Percent inhibition in the binding was calculated as described in the text. As shown in Figure 3, when the guinea pig antisera was diluted 1:40, 95% of the binding of monoclonal Ab₁ to GLXA was inhibited by both unabsorbed and absorbed antisera, compared with 15 % for pre-immune serum. In addition, the unabsorbed antisera had nearly identical inhibition as absorbed antisera at equivalent protein concentration, indicating that no anti-idiotypic antibodies were removed by absorption. Therefore, a competitive anti-idiotypic antibody had been generated in guinea pigs.

Profile of subclasses of guinea pig anti-monoclonal GLXA-Ab₁ IgGs

Previous experiments had shown that different isotypes of guinea pig antiidiotypic exert different effects on the idiotypic production. IgG₁ isotype enhances the idiotypic production, whereas IgG₂ isotype inhibits the idiotypic clone. To test the inhibition of different isotypes of guinea pig anti-idiotypic IgG, subclasses of IgG were isolated from absorbed guinea pig anti-idiotypic antisera. A step pH gradient of phosphate-citrate buffer was used. IgG₁ and IgG₂ were separated and isolated on a protein A affinity column. The elution profile of the isotypes, IgG₁ and IgG₂ is shown in Figure 4. The purification of IgG was accomplished by protein A affinity chromatography. Separation of the guinea pig IgG₁ and IgG₂ was carried out by a step pH gradient of phosphate-citrate buffer. Each peak was identified by immunoelectrophoresis (IEP) with goat anti-guinea pig IgG to immuno-precipitate bands (Bethyl, TX). The first peak which is composed of IgG₁ was precipitated by both goat anti-guinea pig IgG₁ and IgG₂, but formed a single band by goat anti-guinea pig antisera. The first peak was then repurified by the same method.

Inhibition of the binding by subclasses of guinea pig IgG

The competition by different subclasses of IgG was carried out again by chemiluminometric immunoassay using guinea pig anti-idiotypic antibodies of either IgG₁ or IgG₂ isotypes. Figure 5 shows that 0.4 ug IgG₁ was able to inhibit the binding of monoclonal GLXA-Ab₁ to GLXA by 50 %. As shown in Figure 5 Inhibition of the binding of mAb₁ to GLXA by guinea pig anti-idiotypic isotypes. The binding of mAb₁ to GLXA was inhibited by guinea pig anti-idiotypic IgG₁ (•) and partially by IgG₂ (■) in chemiluminometric immunoassay. Homologous unconjugated mAb₁ (○) or PBS (□) were used as controls. Percent inhibition in the binding was calculated as described above. The total inhibition

occurred when 100 ug was used. This was essentially the same concentration needed when unlabeled monoclonal GLXA-Ab₁ IgG was used. IgG₂ showed little, if any, inhibition, approximately 25 % of the binding of monoclonal GLXA-Ab₁ to GLXA with 1.0 ug. These data demonstrate that the IgG₁ subclass of anti-idiotypic IgG was at least 5 times more inhibitory than the IgG₂ subclass.

Generation of Anti-anti-idiotypic Antibodies (GLXA-Ab₃) in Rabbits by Guinea Pig IgG1

The evidence obtained showed that guinea pig anti-idiotypic IgG1 was against the hypervariable region of monoclonal GLXA-Ab₁ IgG and also inhibited its binding to GLXA. The final criteria of an internal image of the anti-idiotypic antibodies is to confirm structurally that the Ab₂ is Ab₂, not Ab₂ or Ab₂. Since Ab₂ binds to the framework portion of immunoglobulins, it can also inhibit the binding of Ab₁ to the cognate antigen. To confirm that guinea pig anti-idiotypic IgG1 is Ab₂, the isotype IgG1 was used to produce an anti-anti-idiotypic antibody (GLXA-Ab₃) which can recognize the GLXA epitope in an animal which has never been exposed to GLXA antigen, (Ertl et al, Proc. Natl. Acad. Sci. USA 81:2850, 1988). Three New Zealand white rabbits were immunized with guinea pig anti-idiotypic IgG1 in the presence of adjuvant, Maalox (alum). The antisera from one rabbit (S2) were tested against IgG1 by ELISA (Fig. 6). The titer increased with time after the immunization. The titer was much higher than 20,000 compared with pre-immune sera three weeks after the boost.

Anti-anti-idiotypic antibodies from rabbits recognize GLXA

The dot blot apparatus (Bio-Rad Laboratories, CA) was used to detect the reactivity of rabbit antisera to GLXA. Antisera from two rabbits were tested by immuno-dot blot assay. Since monoclonal GLXA-Ab₁ is cross-reactive to chlamydial LPS, polyvinylidene fluoride (PVDF) membrane was coated with both

GLXA and chlamydial rLPS. Both rabbits antisera recognized GLXA and LPS with high reactivity. When IgG from rabbit (S2) (9OMS699) was used, the dots were positive at a concentration of 0.006 ug per lane. Pre-immune IgG did not react. The IgG isolated from this antisera was tested for its capacity of inhibiting the binding of monoclonal GLXA-Ab₁ to GLXA, since GLXA-Ab₃ produced by the internal image, GLXA-Ab₂ should exhibit a similar binding. This is confirmed as shown in Figure 7. The inhibition by GLXA-Ab₃ increases with concentration, but about five times less inhibitory compared to unlabeled monoclonal Ab₁. This demonstrates that the guinea pig anti-idiotypic antibody IgG1 is the internal image of antigen, GLXA. GLXA-Ab₃ IgG was further tested for the recognition of elementary bodies of C. trachomatis in vitro.

Monoclonal GLXA-Ab₁ and GLXA-Ab₃ IgG were conjugated with biotin by glutaldehyde. Labeled monoclonal GLXA-Ab₁ or GLXA-Ab₃ was incubated with McCoy cell monolayer on coverslips which were infected with C. trachomatis serovar B (Har 36) elementary bodies for 48 hours. Non-infected monolayers were used as control. The florescence staining pattern of the elementary bodies by monoclonal GLXA-Ab₁ and polyclonal GLXA-Ab₃ IgGs demonstrated that GLXA-Ab₃ not only recognized the purified form, but also native form of GLXA.

Neutralization of the Chlamydial Infection in Primates by GLXA-Ab₃ IgG (9OMS699)

The next question concerned the ability of monoclonal GLXA-Ab₁ or GLXA-Ab₃ to neutralize chlamydia elementary bodies and thus protect host cells from infection. The experimental approaches utilized to answer this question involved neutralization of infection in cultured cells and neutralization of the infection in primate conjunctiva.

In vivo GLXA-Ab₃ neutralizes chlamydial infection whereas monoclonal GLXA-Ab₁ does not.

Neutralization in primate conjunctivae was determined by preincubating organisms with antibody IgG and detection of the effect on ocular infection. The IgG fraction of monoclonal GLXA-Ab₁ or Ab₃ was incubated with elementary bodies of C. trachomatis serovar C. Normal mouse or rabbit pre-immune IgG as well as no immunoglobulin added served as controls. The mixture was inoculated to each eye of the primate. On the day before the and after the inoculation, conjunctival swabs were taken by sweeping different areas of the conjunctivae (see above). Ocular chlamydial infection in primates was determined by cell culture assay which included second-passage on days post-infection. As shown in Table 1, three primates were infected with monoclonal GLXA-Ab₁ treated EBs on the left eyes, GLXA-Ab₃ treated EBs on the right. At the same time, two primates were infected with normal mouse IgG treated EBs (shown as NI) or EBs alone on the left and right eyes alternatively. The same method applied to pre-immune rabbit IgG (shown as N3). All eyes inoculated with monoclonal GLXA-Ab₁ treated EBs were positive at least once (primate No. 515, 84, and 26). However, only one eye of the GLXA- Ab₃ treated eye was positive once, at day 10 post-infection, two of them were never positive (primate No. 515, 84 and 26). Eyes inoculated with normal mouse or pre-immune rabbit IgG treated EBs were all positive at least once (primate No. 563, 20, 17 and 329). The untreated EBs (shown as C) produced infection in two of the four eyes involved (primate No. 563, 20, 17 and 329). Although the data points are diminutive, they do show that monoclonal GLXA-Ab₁ does not neutralize chlamydia in primate conjunctiva. On the other hand, it suggests that GLXA-Ab₃ is neutralizing. In order to confirm that GLXA-Ab₃ does neutralize, a number of tests were carried out, including: (A,) neutralization in cell culture (B), neutralization in primate conjunctiva (C), detection by clinical culture assay (D), detection by direct fluorescence antibody cytology (E), chlamydia specific RNA probe hybridization and (F) determination of

the severity of ocular infection by clinical scoring.

Table 1.
Neutralization of chlamydial infection in primate conjunctivae
by Ab₃ but not mAb₁

Primate	Eye	Ab ^a	Day Following Infection				
			0	3	7	10	14
515	L	mAb ₁	-	-	+	+	-
	R	Ab ₃	-	-	-	-	-
563	L	C	-	-	-	-	-
	R	N ₁	-	-	-	+	-
84	L	mAb ₁	-	-	-	+	+
	R	Ab ₃	-	-	-	+	-
20	L	N ₃	-	-	-	+	-
	R	C	-	-	-	-	-
26	L	mAb ₁	-	-	+	-	-
	R	Ab ₃	-	-	-	-	-
17	L	N ₁	-	-	-	+	+
	R	C	-	-	-	+	+
329	L	C	-	-	+	-	-
	R	N ₃	-	-	-	+	+

^aEBs treated with normal mouse IgG(N1), pre-immune rabbit IgG (N3) or EBs alone (C) were controls.

GLXA-Ab₃ neutralizes the chlamydial infection in vitro.

In vitro, cell culture assay was carried out with GLXA-Ab₃ and pre-immune rabbit antibody. The pre-immune rabbit and GLXA-Ab₃ (9OMS699) IgGs were isolated by protein A affinity chromatography. The mixture of 10 ug of each IgG and 100 ul serovar C EBs (1000 IFU/ml) or EBs alone were inoculated onto wells containing McCoy cell monolayers. Ten wells per sample were used. Inclusion bodies were detected by FITC-conjugated monoclonal anti-chlamydia antibody 48 hours after the incubation. IFU/ml was based on 15 fields per well. As is shown in Table 2, Ab₃ IgG reduced the infectivity 3 times higher compared to pre-immune IgG, 5 times higher compared to EBs alone, indicating neutralization by GLXA-Ab₃.

Table 2.

In vitro neutralization of chlamydial infection by Ab₃ IgG

EBs treated with	Mean IFU/15 Fields + S E M
Ab ₃	34.1 + 7.2
Normal IgG	93.8 + 22.4
None	155.3 + 5.5

GLXA-Ab₃ neutralizes the chlamydial infection in primates

Eight primates were randomly divided into three groups in this experiment. In the eyes, four primates received with purified EBs previously incubated with GLXA-Ab₃ IgG (eight eyes), two received EBs previously incubated with pre-immune IgG (four eyes) and two received untreated EBs (four eyes). On the examining day, the conjunctival swabs were taken and cell cultured. Since there is no significant differences between the recipients of pre-immune IgG and EBs alone, the results are presented as 8 experimental eyes and 8 control eyes. Cell culture results are expressed as IFU/ml based on counting inclusions in 15 fields for two wells per sample. As shown in Table 3, 20 days after the challenge, 1 of eight eyes was

positive compared to eight out of eight eyes that were positive in the control group. When the accumulated results were examined, with GLXA-Ab₃, 9 of 40 (22.5%) were positive, in the contrast, 36 of 40 (90%) were positive without GLXA-Ab₃.

Table 3.

Neutralization of chlamydial infection in primate conjunctivae by
Ab₃ IgG by cell culture assay^a

Day of Experiment	Ab ₃	No. of Eye Culture Positive		
		Preimmune	None ^b	Combined
IgG Control				
0	0/8	0/4	0/4	0/8
2	3/8	2/4	3/4	5/8
6	3/8	4/4	4/4	8/8
9	1/8	3/4	4/4	7/8
12	1/8	4/4	4/4	8/8
20	1/8	4/4	4/4	8/8
	9/40	17/20	19/20	36/40

^a Only one first passage negative sample was second-passage positive.
^bEBs were previously incubated without antibody.

In an parallel assay, direct fluorescence antibody cytology assay (DFA) was also carried out to evaluate the numbers of EBs from the conjunctival swabs. The conjunctival swab samples were fixed onto slides and stained with FITC-labeled monoclonal antibody against C. trachomatis. It was considered to be positive when 5 or more characteristic elementary bodies were seen on each slide (Micro Trak, Syva Co. CA). As shown in Table 4, EBs were detected in the GLXA-Ab₃ treated group on only two occasions, day 6 and 12 post-infection, while the remainder were positive through day 20. Only one eye was EB negative in the non-treated

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group at day 2, that was probably an artifact. EBs were detected in all eyes in this group for the remainder of the experiment. On the last examination day (day 20), none of the eyes treated with GLXA-Ab₃ was positive, while 8 of 8 were positive in the combined control group. In addition, DFA and culture results are completely congruent.

Table 4.

Neutralization of chlamydial infection by Ab₃ using direct
fluorescence antibody cytometry assay (DFA)^a

Day of Experiment	Ab ₃	No. of Eye Culture Positive			
		Preimmune IgG	None ^b	Combined	Control
0		0/8	0/4	0/4	0/8
2		0/8	2/4	0/4	5/8
6		1/8	2/4	4/4	6/8
9		0/8	4/4	4/4	8/8
12		1/8	4/4	4/4	8/8
20		0/8	4/4	4/4	8/8
		2/40	16/20	19/20	35/40

^aDFA was considered positive if 4 EBs were found on a slide.

^bEBs were considered previously incubated without antibody

GLXA-Ab₃ substantially attenuates the chlamydial replication in conjunctival infection

To further understand the mechanism of the neutralization, chlamydial specific ribosomal RNA had been examined from those primate conjunctival swabs by a DNA probe (Cheema et al, The Ameri. J. Med. Sci. 302:261-268, 1991). Total RNA was extracted from conjunctival swabs taken from primate eyes. RNA from serovar C EBs, human or yeast were used as control. ³²P-chlamydial DNA encod-

ing ribosomal RNA16S and 23S genes was used to detect chlamydial specific RNA in a Northern slot-blot hybridization assay. As shown in Figure 8, control eyes (infected either with pre-immune rabbit IgG plus EBs or EBs alone) uniformly show significant levels of chlamydial RNA at all time points examined, similar RNA samples prepared from the eyes of GLXA-Ab₃-treated organism show significantly attenuated levels of chlamydial RNA. This indicates that the neutralization happens at the very early stage of the infection.

The degree of conjunctival inflammation after inoculation with EBs previously incubated with either GLXA-Ab₃ or pre-immune IgG or without previous incubation was evaluated by clinical response. The clinical response was graded based on a total clinical disease scores (TCDS) derived from 10 clinical features of inflammation (Taylor et al, Invest. Ophthalmol. Vis. Sci. 29: 1847, 1988). The accumulative disease scores were obtained for each group of primates by examining 10 signs existing in the conjunctiva. As shown in Figure 9, recipients of GLXA-Ab₃ developed very little clinical disease and this declined after day 8. Control animals continued to develop severe disease through day 21 post-challenge. This pathological finding is consistent with cell culture, DFA and RNA hybridization data.

Generation of And Characterization of Hybridoma Cell Lines Producing Anti-idiotypic Antibody.

Production of anti-idiotypic hybridoma cells

Five syngeneic mice (BALB/cByJ) were immunized intraperitoneally with KLH conjugated monoclonal GLXA-Ab₁ IgG in the presence of Freund's complete adjuvant. The anti-idiotypic anti-sera against monoclonal GLXA-Ab₁ IgG from these mice were detected by sandwich ELISA. The titer was about 1:1000 three weeks after the immunization (data not shown). Fusions were made between Sp2/0-Agl4

cells and spleen cells from two mice which had the highest titer against monoclonal GLXA-Ab₁. The size of the spleens were almost twice the size of normal spleen. The clones were screened by the same method for mouse antisera testing. The pool of mice antisera or pre-immune sera diluted 1:10 was used as controls in all screening tests. Among 283 wells screened, 8 were considered positive against mAb₁ with a titer two times or higher than negative control. These hybridomas were cloned. One clone (91 MS441) had the highest titer in ELISA and was propagated. The ascites was produced and IgG isolated. The isotype of IgG of this clone was identified to be IgG₁, having a K light chain (Bio-Rad Laboratories, CA). The fusion results was summarized in Table 5.

Table 5.

Summary of fusion for anti-idiotypic antibodies

Immunized BALB/cByJ	Anti-id Titer in Supernatant	Total Wells with Growing Hybridoma	Wells Positive of Anti-id (%)	Stable Cloned Anti-id Lines (% of Total)
2	1:1000	283	8 (2.8)	1 (0.35)
	Light chain:K		Heavy chain:IgG ₁	

Hybridoma (91MS441) secreting IgG inhibits the binding of monoclonal GLXA-Ab₁ to GLXA.

The supernatant from positive clones were further tested for the inhibition of the binding of monoclonal GLXA-Ab₁ to GLXA by chemiluminometric immunoassay. Total inhibition of the binding was found from the supernatants of 4 clones without dilution. Two weeks later only one clone (91 MS441) was found stable. As shown in Figure 10, at a dilution of 1:10, the supernatant from this clone inhibits the binding of monoclonal GLXA-Ab₁ to GLXA by 80% compared to 0% of a non-positive clone (91 MS442) from the same fusion. Serial dilutions of culture supernatant from clone 91MS441 (•) and negative clone 91MS442 (O) were tested by chemiluminometric immunoassay. The percent is obtained from the mean of the duplicate. this demonstrates that an anti-

idiotypic clone was produced. It is identified as monoclonal GLXA-Ab₂. Monoclonal GLXA-Ab₂ is recognized by patient and rabbit anti-chlamydia antisera.

As an internal image of an antigenic determinant, the anti-idiotypic antibody should be recognized by any specific antisera which is against this epitope regardless of species. In order to test this property of the IgG produced by clone (91MS441), human serum from a patient clinically diagnosed as chlamydia positive was used. Since the isotype of monoclonal GLXA-Ab₂ is IgG₁, the purification of the IgG was carried out by protein G affinity chromatography. The isolated monoclonal GLXA-Ab₂ IgG was coated on a microtiter plate and tested for reactivity with the patient's serum (91 MS253) by ELISA. Human serum without clinically diagnosed chlamydial infection (88MS356) was used as a negative control. The results showed that the patient's serum had a titer which was more than 1:2000 against monoclonal GLXA-Ab₂ (Fig. 11). The test was carried out by ELISA. Serial dilutions of the antiserum obtained from a patient diagnosed as chlamydial infection (■) and non-diagnosed individual (□) were incubated with mAb₂IgG coated on the polystyrene wells. The second antibody is goat anti-human peroxidase conjugate. Data is presented as the means for duplicates. This demonstrates that infected human antisera has the specific antibody against monoclonal GLXA-Ab₂, implying that monoclonal GLXA-Ab₂ is the internal image of GLXA.

The specific recognition of monoclonal GLXA-Ab₂ by a rabbit anti-chlamydial antisera (88MS 188) was also tested in the similar way. The rabbit antiserum was produced by inoculating EBs. As shown in Figure 12, rabbit antisera has higher binding to monoclonal GLXA-Ab₂ than pre-immune serum, but lower than expected. The difference between the binding is seen to the dilution factor of 1:1000. Polystyrene plate was coated with mAb₂IgG, 1 ug/well. Serial dilutions of rabbit antiserum specific to chlamydial EBs (■) or pre-immune rabbit serum (□) were added to each well in duplicate. The specific binding of the antiserum was detected by mouse anti-rabbit IgG (H&L) HRP conjugate and TMB substrate. This shows that rabbit antiserum which is

against chlamydia has the specific antibody recognizing monoclonal GLXA-Ab₂, an additional evidence for monoclonal GLXA-Ab₂ as an internal image.

Antisera monoclonal GLXA-Ab₃ from syngeneic mice recognize GLXA

The anti-idiotypic antibody as an internal image can raise antigen specific antisera in animals that have never been exposed to that antigen. In this case, if monoclonal GLXA-Ab₂ (91 MS441) is the internal image of GLXA, (that is bearing

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the same antigenic structure), it should produce an anti-GLXA antibody in the same strain BALB/c ByJ mice in spite of the fact that they have never been exposed to GLXA. In an initial experiment, 8 mice were immunized with monoclonal Ab₂ IgG, four mice with normal mouse IgG. No adjuvant was used in the subcutaneous injection. Seven days and 14 days after the immunization and one boost, antisera were obtained from these mice by sacrificing half of the mice in each group. Immuno-dot blot assay was carried out with purified GLXA. The dot blot showed that mice immunized with monoclonal GLXA-Ab₂ IgG had a titer as high as 1:800 compared to 1:100 or less of the mice immunized with normal mouse IgG. The blots were further quantified by a densitometer. When diluted to 1:100 after the first boost, the antisera from all four mice bind to GLXA whereas no binding was seen in the control group even when diluted 1:50. This demonstrates that this antisera is solely elicited by the paratope of the anti-idiotypic because the recipients are syngeneic.

GLXA-Ab₃ from syngeneic mice inhibits the binding of monoclonal Ab₁ to GLXA.

GLXA-Ab₃ antisera which specifically bind to GLXA were also tested for the inhibition of the binding of monoclonal Ab₁ to GLXA by chemiluminometric immunoassay. At a dilution of 1:25, GLXA-Ab₃ inhibits 90% of the binding compared to 18 % of inhibition by the control antisera day 8 post immunization. This is equal to the same inhibition demonstrated by 10 ug of unlabeled monoclonal GLXA-Ab₁. The same inhibition was found in the antisera on day 14, one week after the first boost with the same amount (50 ug/mouse) of IgG. It is noted that inhibition percentage dropped from 70% on day 7 to about 50 % on day 14 when diluted 1:100.

Immunization with Monoclonal GLXA-Ab₂ Protects Mice from Chlamydial Infection

BALB/c ByJ mice were used as an ocular chlamydial infection animal model

in this study. The first experiment was carried out with 39 mice. Twenty mice were immunized subcutaneously with 50 ug of monoclonal GLXA-Ab₂ and nineteen with normal mouse IgG without any adjuvant. They were given a total of three injection, one week apart. One week after the last injection, the mice were inoculated with C. trachomatis serovar C elementary bodies (5000 IFU in each eye). The conjunctival swabs were taken from each eye of the mice on day 0, 7, 14, and 21 post infection. Samples from conjunctival swabs were collected and cultured in McCoy cell monolayers for 48 hours. Inclusion bodies were counted, and 5 inclusions were considered as positive in 15 fields. As shown in Figure 13, mice immunized with monoclonal GLXA-Ab₂ had half the infectivity compared with mice immunized with normal mouse IgG. In a second experiment (twenty mice in each group), the immunization was repeated in the similar manner. However, they received 10⁶ IFU per eye, 100 times the number of EBs as used in the first experiment. Twenty mice were immunized with mAb₂ (•) and nineteen with normal mouse IgG (O) subcutaneously (50 ug IgG/mouse). they were ocular challenged with C. trachomatis serovar C. EBs after three times immunization in a week interval. Each eye received 5000 IFU. One day before and after the challenge, conjunctival sample was taken from each eye and cell cultured. percentage of positive eyes are based on the number of the positive eye among all the eyes examined in a group. Surprisingly, almost the same protection curve found in the mice (Fig. 14), suggesting that monoclonal GLXA-Ab₂ elicits a vigorous host defense response. After immunization three times in a week interval, twenty mice immunized with mAb₂ IgG (•) and twenty with normal mouse IgG (O) received approximate 5x10⁴ to 5x10⁶ chlamydia trachomatis serovar C. EBs per eye. Conjunctival chlamydial inclusions were tested by cell culture obtained from conjunctival swabs. Values are the means of first and second passage culture results except day 28.

In a third experiment, aluminum hydroxide (alum) was used in the immunization: Ten mice were immunized with monoclonal GLXA-Ab₂ in the presence of Maalox as

adjuvant, eight mice immunized without Maalox and sixteen mice immunized with normal mouse IgG in the presence of Maalox. The immunization procedure is the same as the previous ones. When Maalox was used in the immunization, the titer of the anti-anti-idiotypic antisera in mice was doubled as tested against GLXA in immuno-dot blot. The higher titered antisera in the adjuvant treated mice demonstrate more effective protection in the mice from the ocular infection compared to the ones immunized without alum (Fig. 15). Ten mice were immunized with mAb₂IgG without alum (○) and sixteen with normal mouse IgG plus alum (■). One week after the last boost, mice were ocularly infected and conjunctival infection was detected by cell culture on day as shown. However, mice immunized with monoclonal GLXA-Ab₂ had significantly less infection than those receiving normal mouse IgG plus alum. In addition, the infection was cleared by post challenge day 28. In the fourth experiment, eight mice were immunized with

monoclonal GLXA-Ab₂ and eight with normal mouse IgG in the presence of alum. The immunization and inoculation protocols are the same as before. However, as shown in Figure 16 (A), on day 7, both groups had the same degree of infection, 50 % of the eyes are infected. However, beginning on day 10, a significant difference of the infection is seen between the two groups. For the group immunized with monoclonal GLXA-Ab₂ IgG, on day 14, two out of eight eyes were positive. On day 22, one out of eight. No eye was positive on day 28, showing a clear regression of the infection. In contrast, mice immunized with normal mouse IgG, on day 14, six out of six eyes were positive and continued to be positive until day 42. The number of infected eyes began to fall by day 28 seen in this group is believed to be a natural recovery process. In a final experiment, the mice with completely cleared infection from the above experiment were rechallenged with EBs in order to evaluate whether the immunity was associated with memory. A significant factor in terms of protection is the ability of the immunized mice to clear the organisms from their eyes much earlier than the non-immunized ones. This is clearly shown to be the case with mice immunized with monoclonal GLXA-Ab₂ (Figure 16 (B)). This shows that monoclonal GLXA Ab₂ not only is able to evoke a protective immune response but also a memory immune response.

This example illustrates that an anti-idiotypic antibody which mimics GLXA, protects mice as an animal model from chlamydial ocular infection. The monoclonal antibody mAb₁ (89MS30) which was used to produce anti-idiotypic antibodies was produced by immunization with whole EBs and screened for its reaction to a genus specific antigen. It has been used to identify GLXA and demonstrated specific binding to the polysaccharide portion of GLXA. This antibody was also found cross-reactive to cLPS.

First GLXA and cLPS are distinctly different genus specific antigens. GLXA was found on RBs, EBs, inclusion membranes, host cell membranes and shed into the inclusion space, cytoplasm of the infected cells and into the surroundings. It was obtained from the supernatant of infected cell culture. Whereas cLPS was

found both on EBs and RBs (mainly RBs). They are not secreted or shed from infected cells, but loosely bound to the RBs. Structurally, they have different polysaccharide moieties. GLXA has a unique sugar residue: gulose or gulose derivatives, mannose and galactose, probably arranged in repeating units of guluronic and mannuronic acids. Only two fatty acids were found associated with the antigen compared to at least 12 in cLPS. Whereas cLPS has typical linear 2-keto-3-dexyoctonoic acid (KDO) trisaccharide (common core) and polysaccharides like glucosamine and heptose. Serologically, monoclonal GLXA-Ab₁ specifically bind to gulose and mannose repeating block. Whereas, cLPS genus specific epitope is on KDO, the specific determinant is the 2-8 linkage. Obviously, it is very unlikely that GLXA and cLPS share the same epitope. It is known that antibodies, whether poly-or monoclonal, antibodies produced either by whole EBs or by purified cLPS which are specific to cLPS have no neutralizing or protective functions. From the composition analysis, it is suggested that gulose together with mannose and galactose form the specific epitope of GLXA. Whereas, cLPS, in addition to KDO trisaccharide as an epitope, has other epitopes in KDO region and also other saccharide portions. These later structures have shown a broad cross-reaction with LPS from other gram negative bacteria. Since the protective epitope of GLXA consists of an array of sugar residue, it is more reasonable to believe that some of which cLPS is partially shared with GLXA.

There are some other possibilities about this cross-reaction. For example, (1) GLXA and cLPS do not share any primary similarity, but structurally form similar binding motif; (2) although monoclonal GLXA-Ab₁ binds to cLPS, GLXA and cLPS do not have the similarity in antibody binding site. The monoclonal antibody can be multispecific, that is, it recognizes a quite different epitope.

From the discussion above, it is believed that monoclonal GLXA-Ab₁ is specific to GLXA epitope, the possibility that GLXA and cLPS share same sugar residues or merely structure similarity may explain the cross-reactivity.

Anti-idiotypic antibodies, GLXA-Ab₃ and monoclonal GLXA-Ab₂

Anti-idiotypic antibody is a potent and long lived immunogen

Monoclonal GLXA-Ab₁ was injected into guinea pigs subcutaneously in the absence of conjugate or Freund's adjuvant. All four immunized guinea pigs developed high titered anti-idiotypic antibodies specific to monoclonal Ab₁, which were found as early as 3 weeks after the first immunization. The titer was approximately 1:5000 by ELISA. The production of the anti-idiotypic antisera contain a relatively high concentration of GLXA-Ab₂ which is specific to the hypervariable region of monoclonal GLXA-Ab₁. This was shown after two absorptions by normal mouse IgG. When the IgG1 isotype from guinea pig anti-idiotypic antibodies was used as an immunogen in three rabbits, the titer of anti-anti-idiotypic antibody was more than 1:20,000 two months after immunization. This demonstrates that immunoglobulin itself is a very potent immunogen. This is true not only for interspecies immunization, but also syngeneic immunization. With monoclonal anti-idiotypic antibody monoclonal GLXA-Ab₂, the immunization was carried out in syngeneic mice without KLH-conjugation or Freund's adjuvant. The mice developed high titered GLXA-Ab₃ in a short time after immunization (9 days). The protocol used in this study is different from most methods which use either a conjugate or Freund's adjuvant for a higher immunogenicity. This indicates that immunoglobulin as an antigen is more immunogenic compared to most isolated or synthetic peptide antigens.

A successful vaccine not only requires that it be a good immunogen but that it is long lasting (preferably for the lifetime off the host). The immunity produced by idioype is long lived. The ability to inhibit the binding of monoclonal GLXA-Ab₁ to GLXA by guinea pig GLXA-Ab₂ from three immunized guinea pigs have been monitored for as long as 77 weeks. With only three boosts, the inhibition one year post immunization is almost equal to antisera collected in the early stages after the immunization. This indicates that the immunity elicited by the idiotypic antibody

monoclonal GLXA-Ab₁ has a long term memory. Since the half-life of an antibody molecule or the majority of antibody-producing cells is about a few weeks, the boosting interval (six months) is far beyond the life span of the B cells and the immunoglobulins. It is the constant stimulation within the idiotypic network that keeps this anti-idiotypic antibody at a certain level. The change of idiotype specificity during this period has not been seen in this case.

An internal image of chlamydial GLXA, isotypic difference

In this study, guinea pig GLXA-Ab₂ IgG1 and IgG2 were separated. The regulatory function of these two isotypes to the idio type was not evaluated. However, the difference between IgG1 and IgG2 subclasses have been found in inhibition of the binding of monoclonal GLXA-Ab₁ to GLXA. With a novel system, chemiluminometric immuno-assay, the incubation and the final detection were all carried out in solution rather than solid phase as in ELISA, thus greatly lessening the possibility of the inhibition by hindrance. The results have shown that GLXA-Ab₂ IgG1 inhibited 100% of the binding whereas IgG2 50% at the same concentration. This suggested that GLXA-Ab₂ IgG1 has a high affinity in binding to the idio type or being more like the antigen, GLXA. This demonstrates an isotypic difference in their binding ability to the idio type which reflects a difference in their respective active sites. IgG1 has a different idio type binding ability from IgG1. There are a number of examples of dominant idiotypes, for example, A5A idio type of anti-strep-A carbohydrate antibodies or the T15 idio type of phosphoryl choline antibodies. It is not clear if there is any isotype preference of anti-idiotypic antibody in different systems. This finding suggests that a certain isotype of GLXA-Ab₂ is the internal image while others are not.

Monoclonal GLXA-Ab₂ as an immunogen of chlamydial GLXA

The purpose of making monoclonal anti-idiotypic antibodies is to: (1) have a constant source of anti-idiotypic antibody for vaccine study; (2) identify a possible receptor for GLXA on host cells; and (3) further characterize the epitope on GLXA. This enables an understanding of biological functions of GLXA in terms of epitope density, its role in mechanism of infection and the protective function against chlamydial infection in vivo. The production of monoclonal anti-idiotypic antibodies was carried out in the syngeneic BALB/cBYJ mice. In the first fusion, one stable, highly inhibitory clone (91MS441) from 283 clones screened was selected. In the second fusion, another clone (91MS442) was selected though it is not as inhibitory as 91MS441 clone in chemiluminometric immunoassay. The monoclonal GLXA-Ab₂ produced by this clone (91MS441) has been shown to be the internal image of the chlamydial antigen, GLXA.

It is interesting to note that the inhibitory ability of mouse GLXA-Ab₃ slightly but obviously decreases over time. The dosage of the anti-idiotypic has been a factor in either enhancing the idiotypic or suppressing the idiotypic. This inhibition results by GLXA-Ab₃ has shown that after administration of monoclonal GLXA-Ab₂ IgG twice, the inhibition is higher than the sera obtained after administration three times. This indicated 50 ug is either too much for one dose or too much for repeated administrations. On the other hand, it shows that a low amount is enough for protective immunity. The reason for choosing normal mouse IgG as a negative control in the immunization rather than a non-relative clone is that it would prevent any possible bias from a specific clone.

Monoclonal GLXA-Ab₁ and GLXA-Ab₃ bear the same antigen binding structure.

GLXA-Ab₃ from immunized rabbits and mice recognized affinity purified GLXA by immuno-dot blot assay, though there is no previous exposure to GLXA or infection. The binding of monoclonal GLXA-Ab₁ to GLXA is inhibited as the concentration of GLXA-Ab₃ increases. This indicates that the anti-anti-idiotypic has equivalent antigen reactivity as idiotypic, that is they recognize the same

epitope. The same antigen reactivity of monoclonal GLXA-Ab₁ and GLXA-Ab₃ from rabbits to GLXA was further proved in the immuno-fluorescent staining of the inclusions in infected McCoy cell culture. The experiment suggested the structural similarity of antibodies. Using monoclonal GLXA-Ab₂ which mimic GLXA to identify its role would be valuable in understanding the mechanism of anti-idiotypic protection. The experiment was carried out with human epidermoid carcinoma cells (A431) as well as human endometrial gland epithelial cells (HEGEC). Those cell lines were used because of their human origin. A preliminary binding experiment was performed by direct antibody staining detected with FACScan (Becton Dickinson, N.J.) flow cytometer. Since the separation of these epithelial cells into a single cell suspension is very difficult without using enzyme or harsh separation, especially HEGEC cells, the cell population consists of singlets, doublets and multiplets. Single cell population was gated right above the cell debris. Since the control, cells stained with normal mouse IgG was at exactly the same gate, it is believed that the two groups are comparable. In addition, the binding is direct, monoclonal GLXA-Ab₂ or normal mouse IgG was directly biotin labeled. The intensity of monoclonal GLXA-Ab₂ bound increases with the concentration specifically to the host cells (HEGEC). While normal mouse IgG has no such binding even at the highest concentration. If this finding can be repeated and proved to be valid, GLXA is an adhesin or a ligand which binds to HEGEC cells. There are some reasons to believe that GLXA is an adhesin or a ligand. First, as mentioned above, GLXA-Ab₃ neutralization occurs at the very early stage. This is proved by finding apparently no significant chlamydial specific ribosomal RNA in conjunctiva samples taken from primates inoculated with GLXA-Ab₃ treated EBs. This suggested that GLXA-Ab₃ blocked EBs from getting into the host. Second, GLXA was found to enhance the infection by approximately three fold when McCoy cells were pre-incubated with GLXA. It is only if GLXA as an adhesin or ligand which facilitate the attachment of EBs to host cells that this result can occur. Actually, EBs may well use this mechanism to infect host cells because GLXA are secreted

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from infected cells.

A possible mechanism of the role of GLXA may be the following: EBs attach to the host cells either by GLXA on EBs or by free GLXA which were secreted by the infected cells into the surroundings. This makes it much easier and efficient for GLXA to absorb to surrounding cells. Ebs can then attach to the host cells either by GLXA on EBs, thus binding to the host cells. This mechanism seems much more efficient than one to one attachment.

Neutralization of chlamydial infection by GLXA-Ab₃

The preliminary neutralization test in vitro has shown that more chlamydial inclusions were found with GLXA-Ab₁ treated than rabbit Ab₃ treated C. trachomatis serovar B EBs. This showed that GLXA-Ab₃ neutralized the infection, whereas monoclonal GLXA-Ab₁ did not. GLXA-Ab₃ neutralized the infection in cell culture whereas monoclonal GLXA-Ab₁ did not. This in vitro result was repeated in vivo in two later experiments with the primate infection model. It was confirmed that GLXA-Ab₃ effectively neutralized chlamydial infection both in vitro and in vivo.

The understanding of the mechanism of this neutralization comes from the chlamydia specific RNA hybridization experiment. The primates were ocularly inoculated with GLXA Ab₃ or normal rabbit IgG treated EBs. Chlamydial RNA was detected from the conjunctival swabs taken from primates on different days prior and post inoculation. The RNA hybridization assay used in this study is an extremely sensitive way to detect chlamydial infection in samples which are unequivocally negative by either cell culture of DFA or both. The substantially low RNA found in Ab₃ treated primates provides the evidence that the neutralization occur at very early stage of the infection, before the internalization of the organisms. This suggests that GLXA may be an adhesin or ligand functioning at the stage of attachment.

The role of humoral immunity in protection against chlamydial infection has long been discrepant. However, it has been shown both in vitro and in vivo that

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humoral immunity may play some role in preventing chlamydial infection.

Protection of Mice from chlamydial infection by immunization with monoclonal GLXA-Ab₂

The chlamydial ocular infection model in BALB/cByJ mouse was employed in a vaccine study with monoclonal anti-idiotypic antibody monoclonal GLXA-IgG. It is model for C. trachomatis serovars which only infect humans. In the immunization experiments, monoclonal (GLXA-Ab₂) IgG (50 ug/mouse) was used to immunize 6 to 20 mice in each group subcutaneously either in the presence of adjuvant or with no adjuvant. Mice immunized with monoclonal GLXA-Ab₂ mount a distinctive GLXA-Ab₃ titer detectable one week after a single immunization. The system used in this study is syngeneic: monoclonal GLXA-Ab₁, monoclonal GLXA-Ab₂ or GLXA-Ab₃ are all produced in the same strain of mice. They bear the same immunoglobulins genetically. The only "foreign structure" is the specific binding site of each type antibody (monoclonal GLXA-Ab₁ or monoclonal GLXA-Ab₂). Normal mouse IgG from the same strain was used as a negative control. Consistently, mice immunized with monoclonal GLXA-Ab₂ developed a significantly high GLXA-Ab₃ titer against purified GLXA compared to the control group. The average titer was as high as 1:3200 comparing with 1:200 in the mice immunized with normal mouse IgG. This clearly demonstrates that the production of GLXA-Ab₃ antibody in syngeneic mice is elicited by the hypervariable region, the structure which mimics the GLXA. In addition, the GLXA-Ab₃ from these mice inhibited the binding of monoclonal GLXA-Ab₁ to GLXA as efficiently as unlabeled monoclonal GLXA-Ab₁. These experiments shows that monoclonal GLXA-Ab₂ mimics one single epitope of GLXA.

The protection of mice from ocular infection by C. trachomatis was consistently demonstrated in four individual experiments in vivo. The mice immunized with monoclonal GLXA-Ab₂ or normal mouse IgG (three times on a weekly basis) were infected in the eyes by inoculation of EBs (5000 IFU/eye). The

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conjunctival swabs were taken on different days before and after the infection. Inclusions were detected by cell culture. The eyes of the mice immunized with monoclonal GLXA-Ab₂ IgG have significantly short recovery (9 days after ocular inoculation comparing to 28 days of the control immunized with normal mouse IgG). The GLXA-Ab₃ titer is well correlated with cell culture results. This demonstrates that monoclonal GLXA-Ab₂ which mimics a single GLXA epitope, is an effective immunogen, and evokes a strong protective immune response against chlamydial infection. This shows that a single protective epitope resides on chlamydial GLXA.

Prospective of the mechanism of the neutralization and protection

This example is the first demonstration that a non-neutralizing monoclonal GLXA-Ab₁ can produce a neutralizing GLXA-Ab₃ in vivo through an anti-idiotypic antibody (GLXA-Ab₂). The central issue is that monoclonal GLXA-Ab₁ produced by immunization with chlamydial EBs does not neutralize or protect primates from reinfection whereas GLXA Ab₃ produced by guinea pig GLXA-Ab₂ neutralized the infection and immunization with monoclonal GLXA-Ab₂ protects mice from reinfection.

ORAL VACCINE

(a) Infection of mouse eyes: Eyes of BALB/c mice were infected by topical application of $2500-10^6$ IFU/5 ul of Percoll purified elementary bodies (EB) from serovar C (TW/3) suspended in SPG or PBS. Concentration was reconfirmed by titration at each challenge. The course of infection was followed by clinical exam, microbiologic assays, histopathologic exams, and immunologic assay. Modified grading scales for clinical scoring and histopathology were developed. Conjunctival specimens were collected with sterile urethral swabs and tested in culture or direct fluorescent antibody (DFA) assays for the presence of chlamydial organism by standard methods, Infect, Immun., 1989, Vol 57, Pgs. 2977-2983, with the exception that blind second passages were performed on all specimens.

(b) Test of the anti-idiotypic antibody (Ab_2) as a protective vaccine: BALB/c mice were immunized subcutaneously 1-3 times with either 50 or 100 ug Ab_2 in alumina (Maalox). Ten to 12 days after the last immunization, an Ab_3 response was verified by a chemiluminescent or GLXA dot blot assay, Curr. Microbiol; 1933, Vol.28, Pgs. 885-890, after which all eyes were challenged with infectious C-EB (5000 IFU). The course of infection was followed as described above. Control mice received the same regimen of purified normal ascites IgG_2 and infectious challenge.

Ab_2 or IgG incorporated into biodegradable microspheres was used to immunize BALB/c mice, either orally or subcutaneously, with 50 ug Ab_2 equivalent (w/w). Microspheres were prepared by using as polymer, polylactic acid, Bio/Technology, 1933, Vol. 10, Pgs. 1446-1449 and Pharmaceut. Res., 1991, Vol. 8, Pgs. 713-720. Retention of Ab_2 function was confirmed by the ability of microspheres containing the Ab_2 to induce an Ab_3 response after subcutaneous injection. Control mice received IgG_2 in microspheres, soluble Ab_2 in alumina, or control IgG in alumina. Five-10 mice/group were challenged on day 0 and followed for protection against ocular infection.

Results

(a) A reproducible inbred mouse model of ocular infection by a human biovar.

BALB/c mice developed reproducible ocular infection after single or repeated inoculation with serovar C of *C. trachomatis*. While clinical disease was most evident with repeated infection (daily, repeated weekly or once weekly), even a single inoculation of infectious chlamydia induced lid thickening and exudate. Histopathologically, intensity of inflammatory mononuclear infiltrate loss of goblet cells, and exudate were dose-dependent. The mean histopathologic disease score at day 12-14 was 6.8 ± 0.8 compared to 0 ± 0 for normal tissue. A typical microbiologic timecourse obtained with conjunctival swabs from 10 BALB/c mice is shown in Figure 17.

(b) Soluble anti-idiotypic antibody protects against ocular chlamydial infection.

Ab₂ in alumina successfully immunized mice as evidenced by prechallenge Ab₃ dot blot titers ranging from 1:200-:800 compared to <1:100 for controls. After infectious ocular challenge, conjunctival swabs were collected for culture at days 7, 10, 14, 21, 28, 35 and 42. Culture results from a typical experiment are shown in Figure 18. One hundred percent of control mice (n=10) which received IgG with alumina became infected by day 12-14. In contrast, 50% of Ab₂ recipient eyes were totally protected and those eyes which were culture positive at day 7 cleared infection 2-4 weeks earlier than controls. Histopathologic disease scores were reduced from 9.2 ± 0.8 (IgG) to 2.9 ± 0.8 (Ab₂). A higher infectious challenge dose (10^6 IFU/5ul) or a lower dose (50 ug of Ab₂) protected similar numbers of eyes. Similar protection was observed after rechallenge of animals after they became culture-negative.

(c) Ab₂ in microspheres protects after oral or subcutaneous immunization.

In order to optimize presentation of the anti-Id to the mucosal immune system, experiments were performed with Ab₂ incorporated into biodegradable microspheres. Groups of 5-10 mice recieved 50 ug Ab₂-microspheres either orally (p.o.) in 200 ul bicarbonate buffer, subcutaneously (s.c.) in PBS, soluble Ab₂ in alumina (Maalox) s.c., or oral IgG-microspheres. After one boosting immunization, all mice received an infectious ocular challenge of 5000 IFU C-EB. On day 12, all eyes were swabbed for culture assay. The results are shown in Table 6. Microbiologic protection was highly significant in recipients of Ab₂-microspheres based on reduced mean inclusions per group compared to recipients of IgG-microspheres ($p < .003$). Second passage outline confirmed first passage results.

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Table 6Test of Ab₂ in Microspheres as an anti-Chlamydial Vaccine

Grp-Treatment (#mice)	Passage 1 (%Positive/Grp)			Passage 2% Positive Grp)		
	% Mice	Mean IFU	P	%Mice	Mean IFU	P
A:ORAL Ab ₂ -Microspheres (n=8)	75	0.73±0.3	(<.002)	75	1.47±0.4	(<.003)
B:S.C. Ab ₂ -Microspheres (n=10)	50	0.94±0.4	(<.01)	70	1.18±0.4	(<.001)
C:S.C. Ab ₂ -in Maalox (n=5)	80	0.66±0.3	(<.006)	100	1.15±0.5	(<.004)
D:ORAL IgG-Microspheres (n=6)	100	3.0±0.5		100	4.21±0.6	

All animals received 50 ug Ab₂ or IgG twice; P-values in comparison to Group D.

This example demonstrates that inbred BALB/c mice can be used as a model of ocular chlamydial infection. A single infectious inoculation of a human biovar of *C. trachomatis* (C/TW-3) leads to ocular infection in BALB/c and C3H/HeN mice which can be detected clinically, histopathological, microbiologically, and by immunoassay.

The monoclonal anti-idiotypic antibody (mAb₂) to anti-GLXA mAb₁ was tested as a potential anti-chlamydial vaccine candidate in the mouse model. mAb₂ competes with mAb₁ for binding to GLXA and immunization with mAb₂ induces Ab₃ which recognizes the same epitopes on GLXA as recognized by mAb₁. The animals immunized with mAb₂ had no previous exposure to GLXA or whole chlamydia, yet the Ab₃ induced by mAb₂ reacted with GLXA in dot blot and ELISA essays.

The results demonstrate unequivocally that Ab₂ can successfully immunize against ocular chlamydial infection, even after systemic immunization with soluble anti-Id in alumina. Ab₂ incorporated into microspheres protected 50% of eyes from

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infection after systemic immunization, and 44% of eyes after oral presentation of the same dosage. The mean inclusion counts for recipients of Ab₂- microspheres were reduced by 65-72% compared to IgG-microsome controls. The results strongly support the usefulness of an anti-idiotypic antibody as an anti-chlamydial vaccine and demonstrate the feasibility of Ab₂ incorporated into microspheres. In addition to being highly immunogenic, protection by the criteria of reduced clinical, histopathologic, and microbiologic disease were also fulfilled for both soluble Ab₂ and Ab₂ delivered in microspheres.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An oral vaccine for administration to an animal in an amount sufficient to effect immunization against chlamydia which comprises microspheres having a solid matrix formed of a pharmacologically acceptable polymer containing a biologically active composition, wherein said composition is selected from the group consisting of a monoclonal anti-idiotypic antibody, a Fab fragment of said monoclonal anti-idiotypic antibody, a (Fab)₂ fragment of said monoclonal anti-idiotypic antibody, and mixtures thereof, wherein said monoclonal anti-idiotypic antibody, said Fab fragment and said (Fab)₂ fragment induces in an animal anti-anti-idiotypic antibody which binds an epitope on genus specific chlamydia glycolipid exoantigen (GLXA).
2. The vaccine of claim 1 wherein said biologically acceptable composition is a Fab fragment of a monoclonal anti-idiotypic antibody.
3. The vaccine of claim 1 wherein said biologically acceptable composition is a (Fab)₂ fragment of a monoclonal anti-idiotypic antibody.
4. The vaccine of any one of claims 1, 2 or 3 wherein said polymer is polylactic acid.

5. The vaccine of any one of claims 1, 2 or 3 wherein said polymer is poly(lactide-co-glycolide).
6. The vaccine of claim 1 wherein said monoclonal anti-idiotypic antibody is provided from the continuous hybrid cell line, ATCC Acc. No. HB 11301.
7. The vaccine of claim 1 wherein said Fab fragment is produced from a monoclonal anti-idiotypic antibody provided from the continuous hybrid cell line, ATCC Acc. No. HB 11301.
8. The vaccine of claim 1 wherein said (Fab)₂ fragment is produced from an antibody provided from the continuous hybrid cell line, ATCC Acc. No. HB 11301.
9. The vaccine of claim 1 wherein said animal is a human.
10. The vaccine of claim 2 wherein said animal is a human.
11. The vaccine of claim 3 wherein said animal is a human.
12. Use of a biologically active composition which comprises microspheres having a solid matrix formed of a pharmacologically acceptable polymer, wherein said composition is selected from the group consisting of a monoclonal anti-idiotypic antibody, a Fab fragment of said monoclonal anti-idiotypic

antibody, a (Fab)₂ fragment of said monoclonal anti-idiotypic antibody, and mixtures thereof, and wherein said monoclonal anti-idiotypic antibody, said Fab fragment and said (Fab)₂ fragment induces in an animal anti-anti-idiotypic antibody which binds an epitope on genus specific chlamydia glycolipid exoantigen (GLXA) in an amount sufficient to effect immunization against chlamydia infection, for oral immunization of an animal against chlamydia infection.

13. Use according to claim 12 wherein the monoclonal anti-idiotypic antibody is produced by continuous cell line ATCC Acc. No. HB 11301.

14. Use according to claim 12 wherein said animal is a human.

15. Use according to claim 13 wherein said animal is a human.

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PATENT AGENTS

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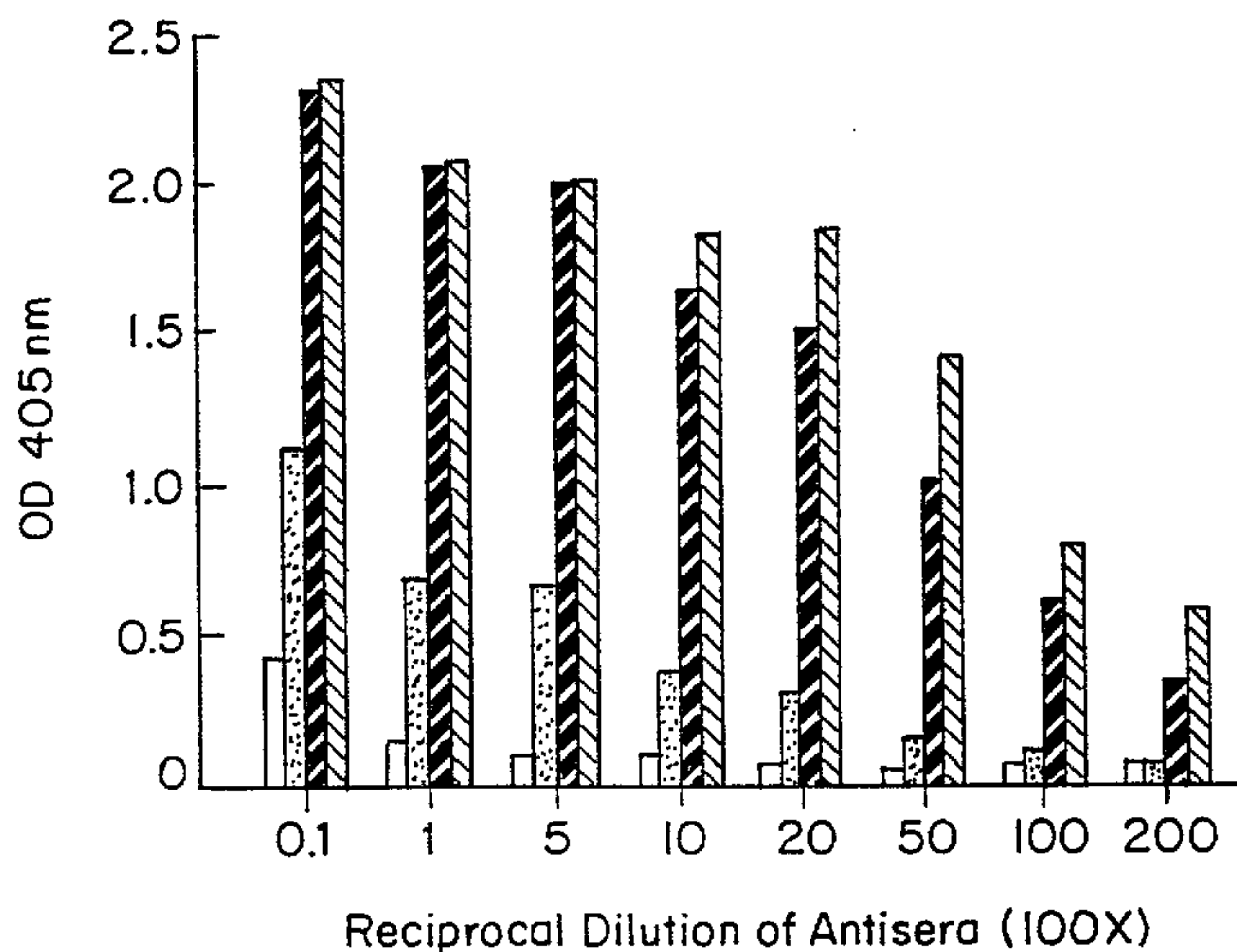


FIG. 1

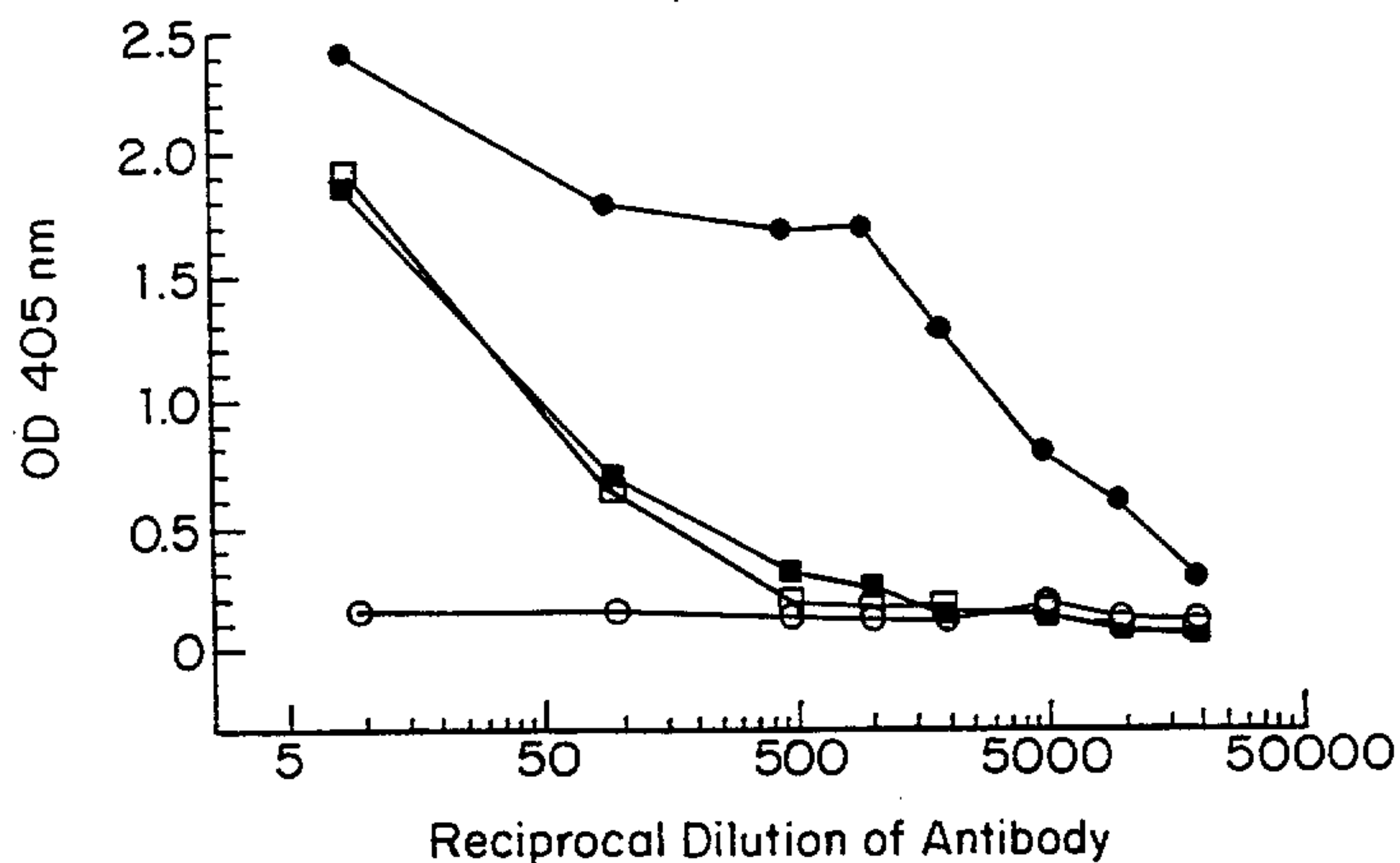


FIG. 2

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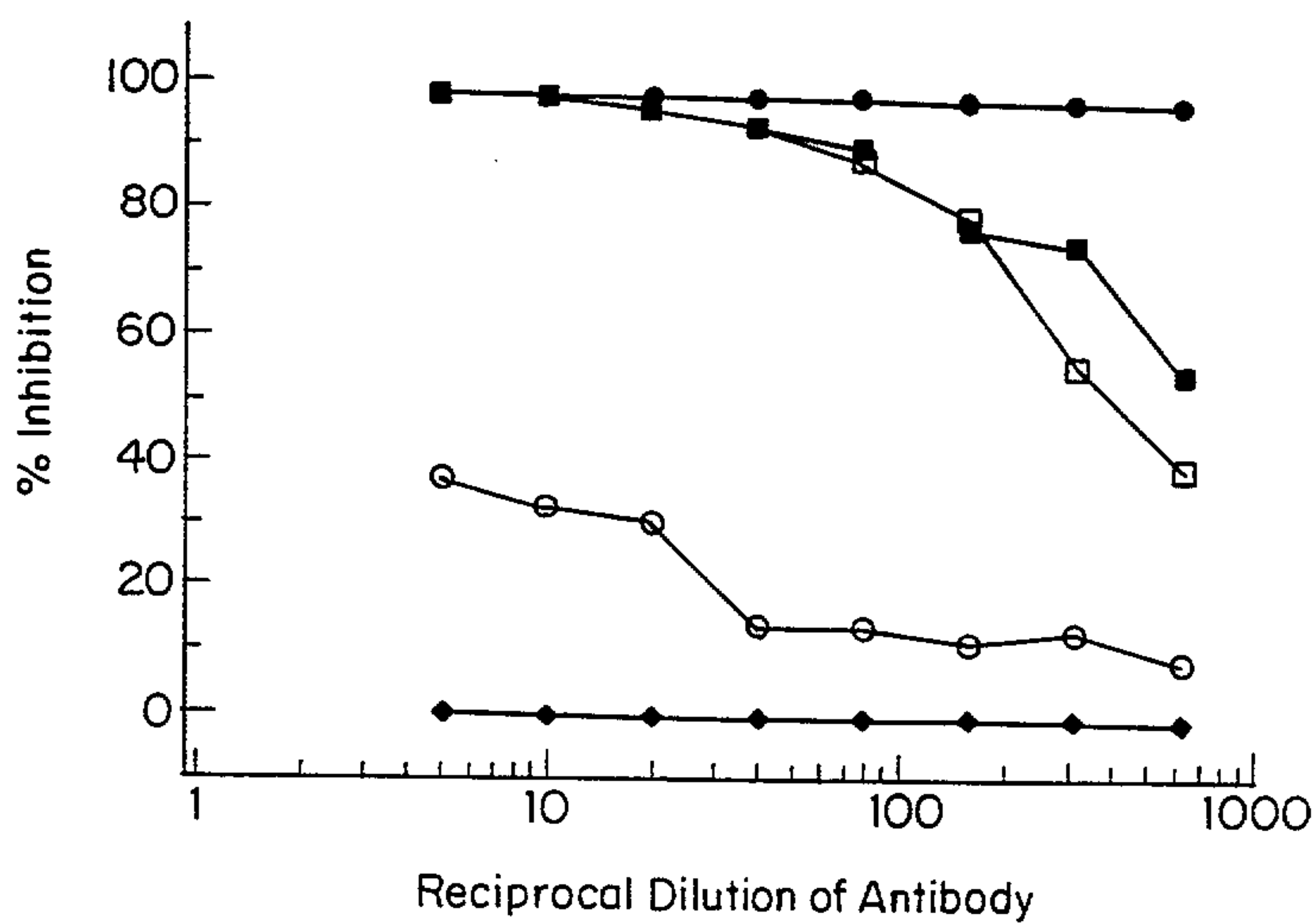
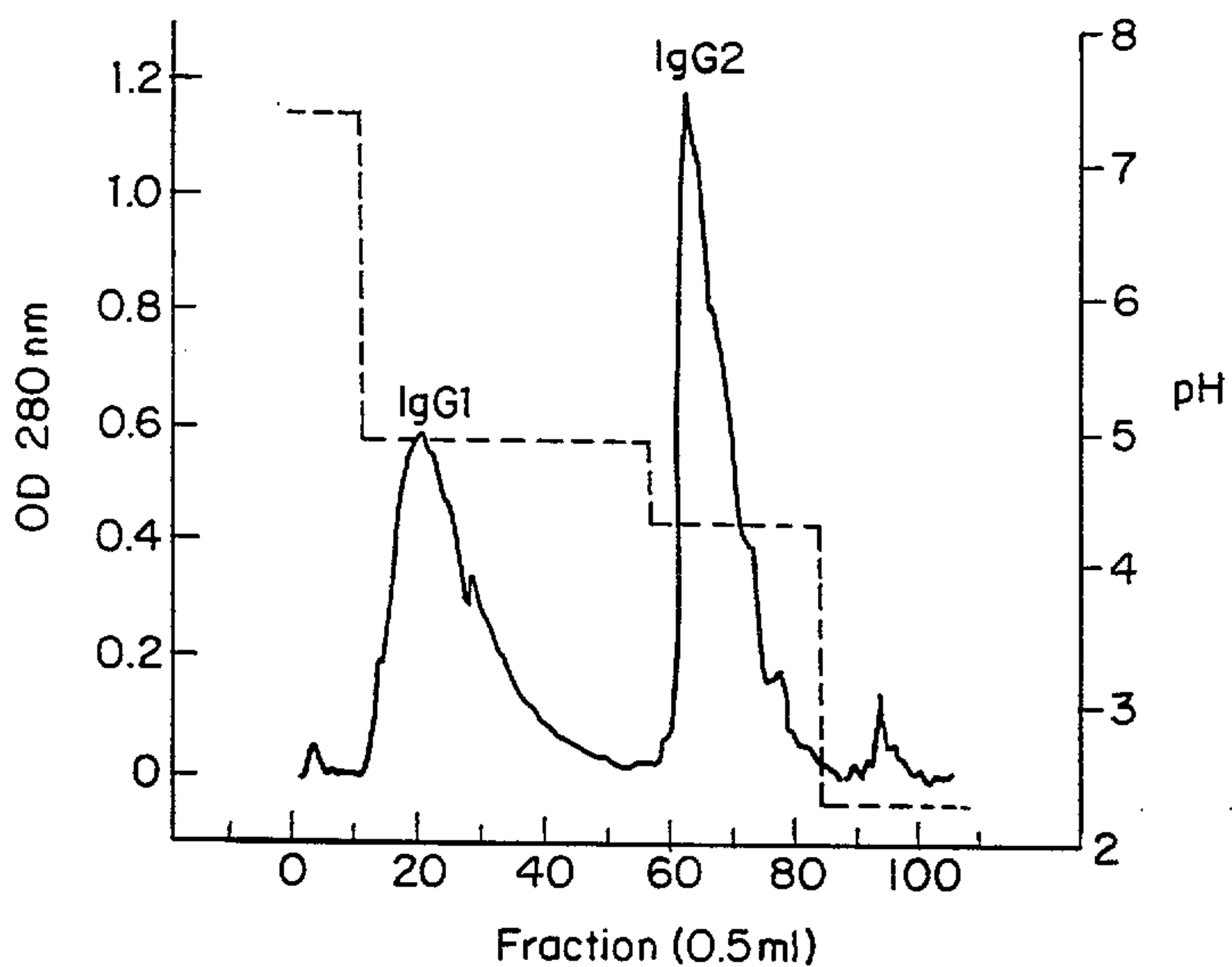


FIG. 3

FIG. 4
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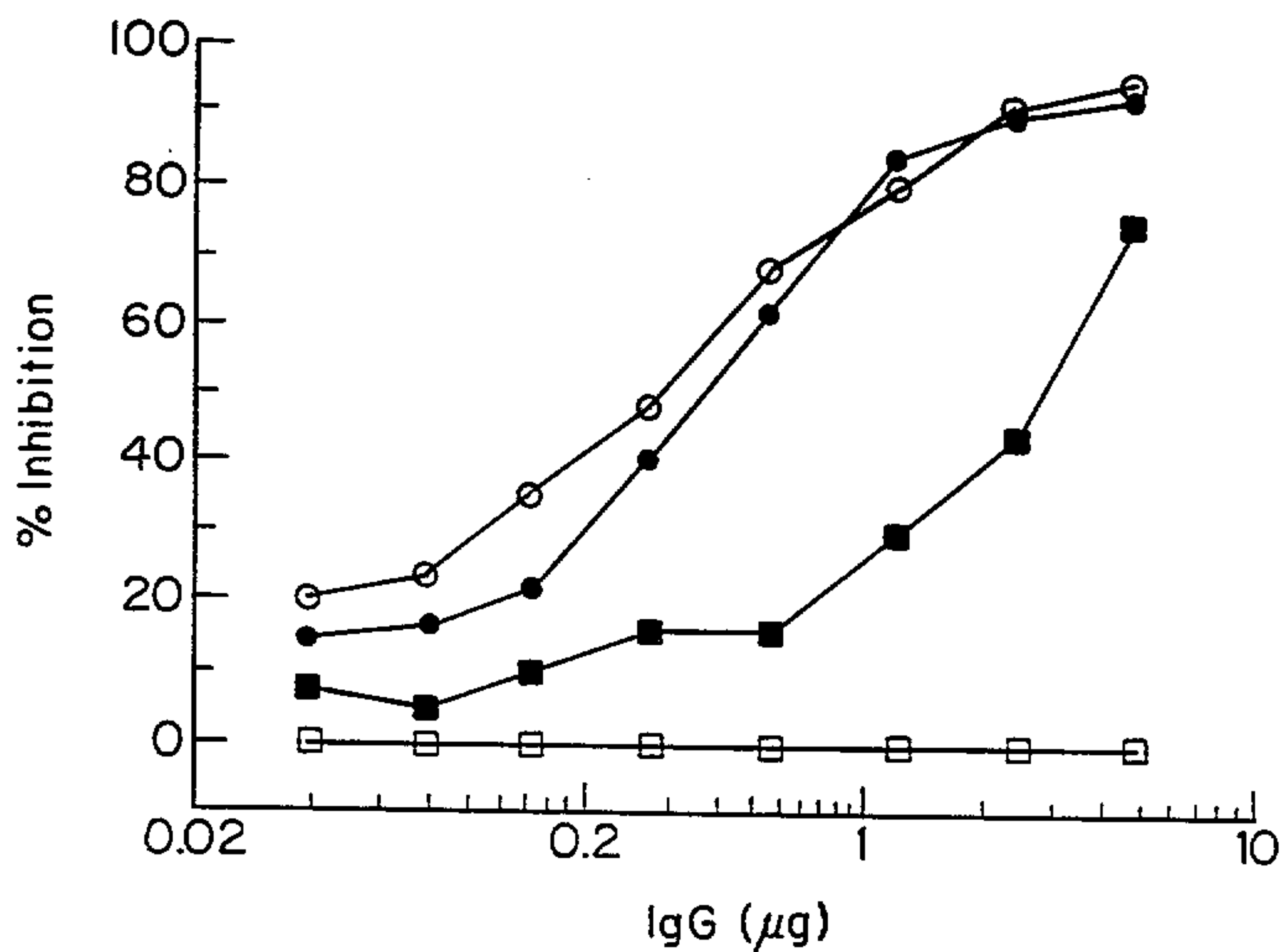


FIG. 5

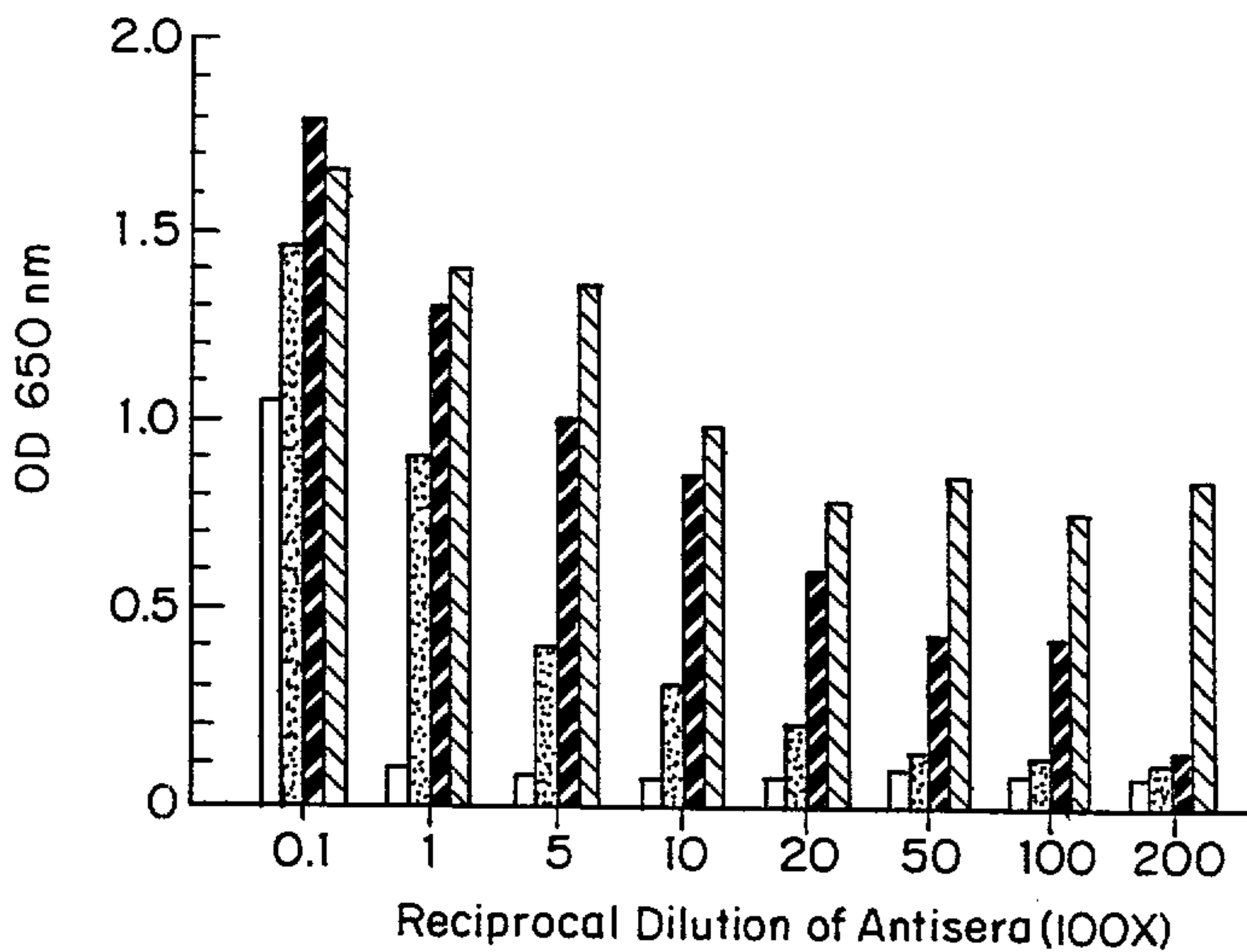


FIG. 6

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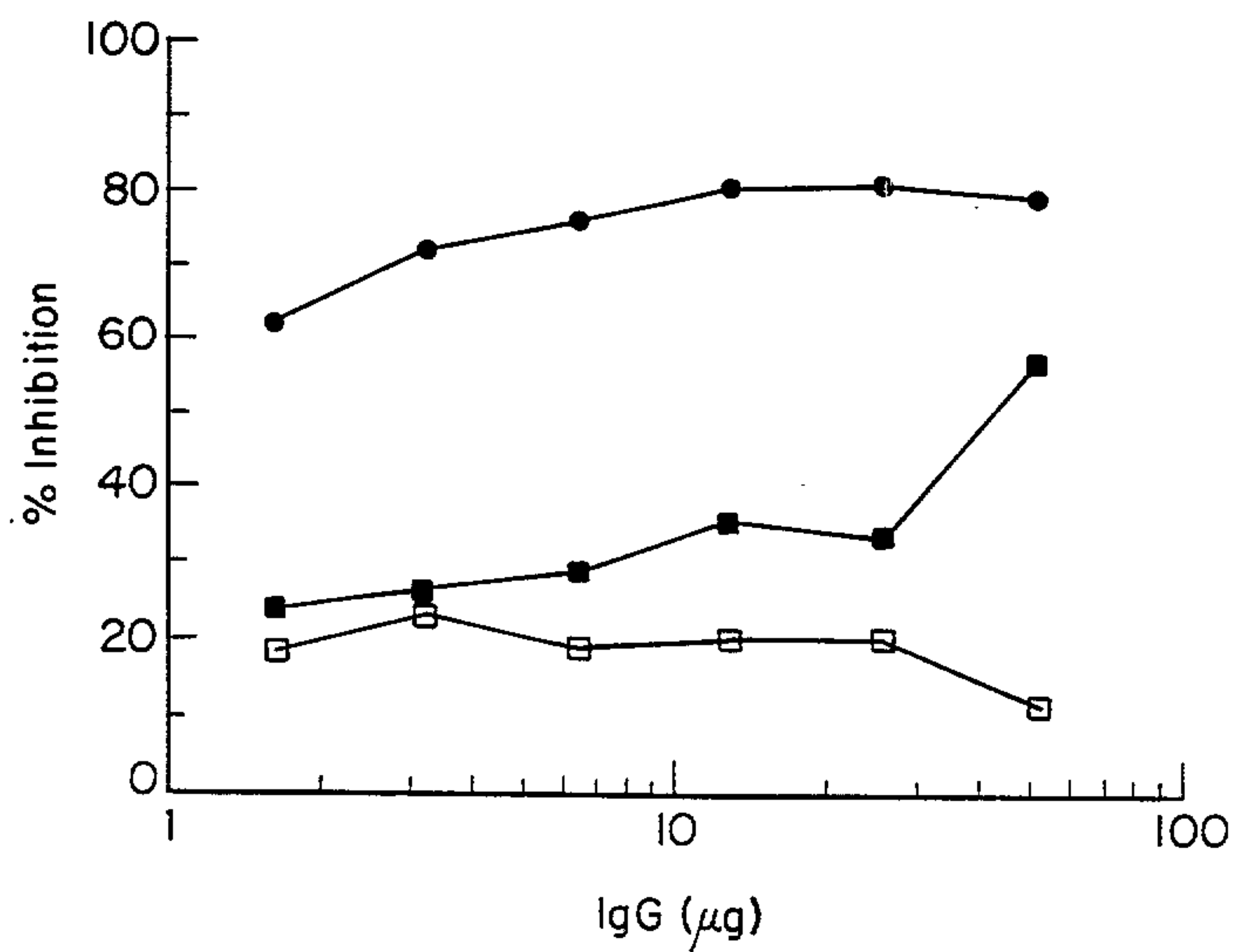


FIG. 7

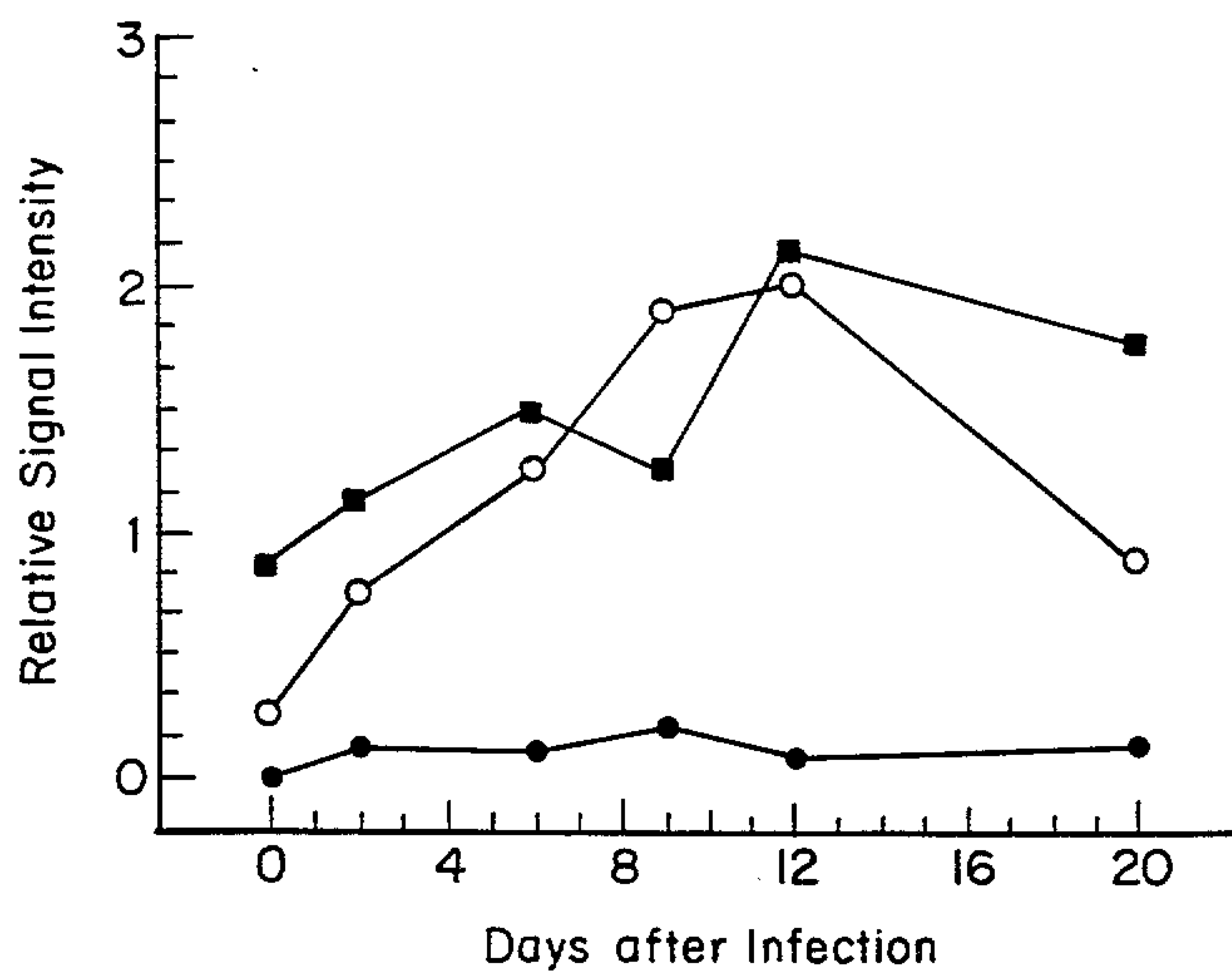


FIG. 8

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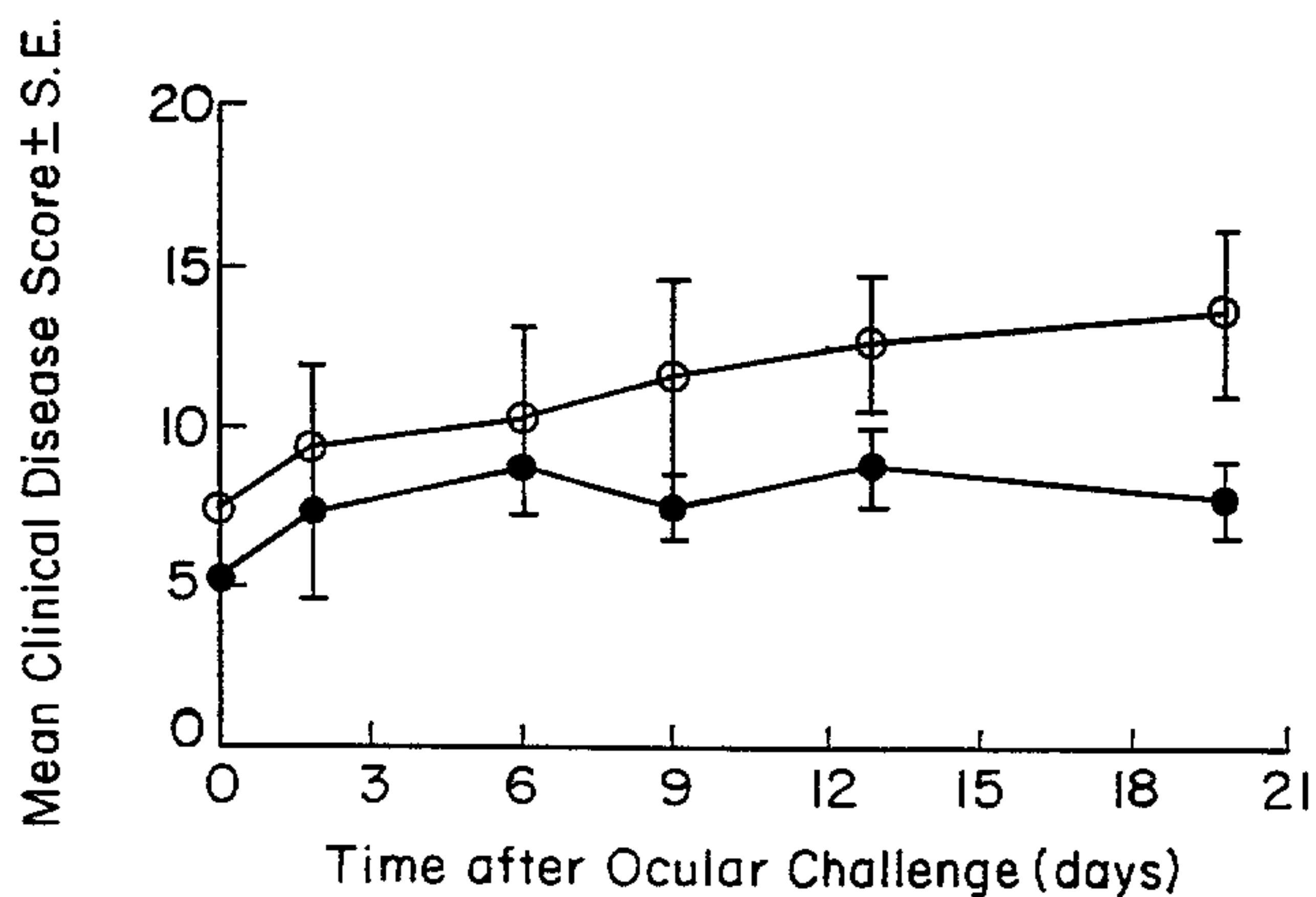


FIG. 9

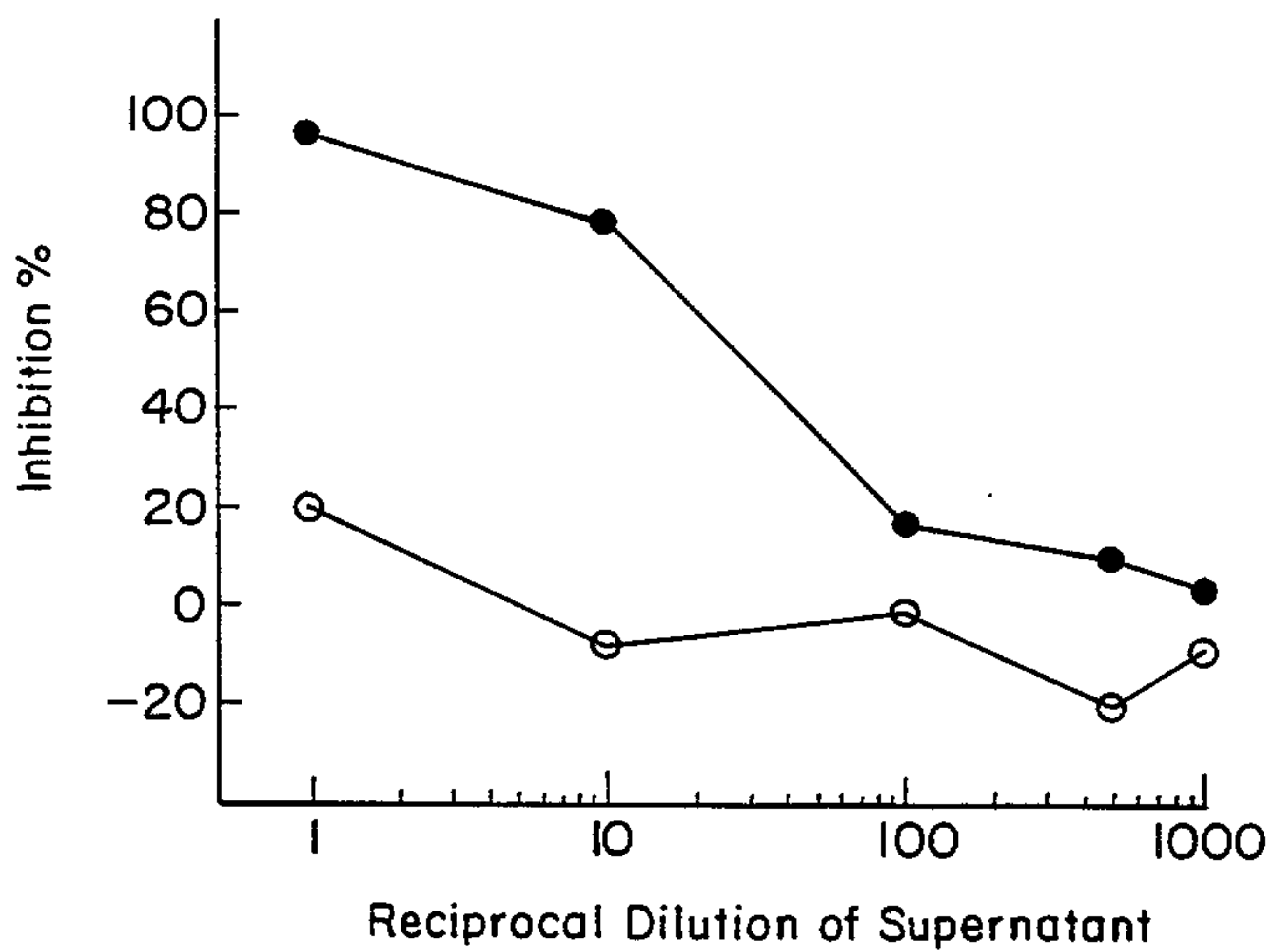


FIG. 10

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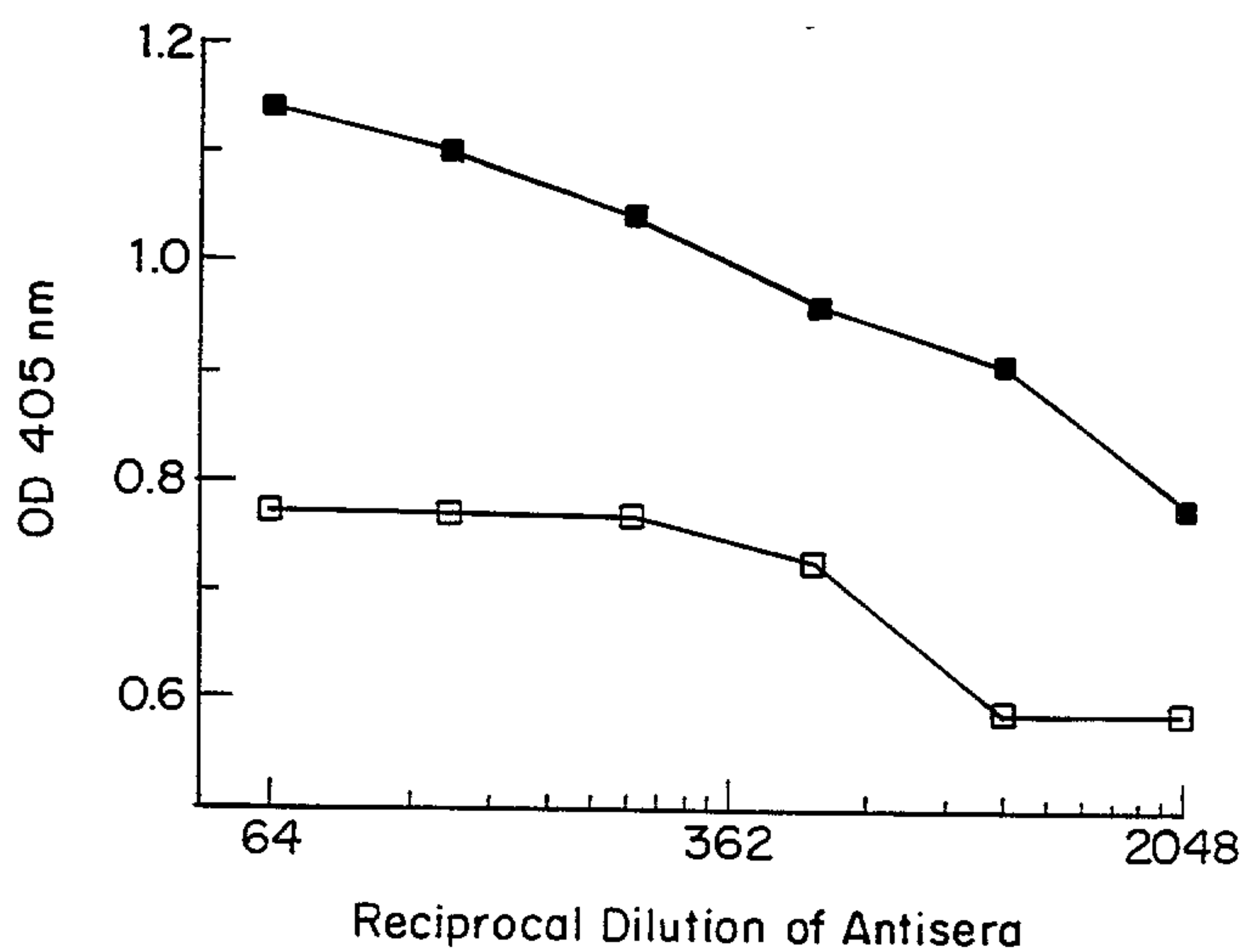


FIG. 11

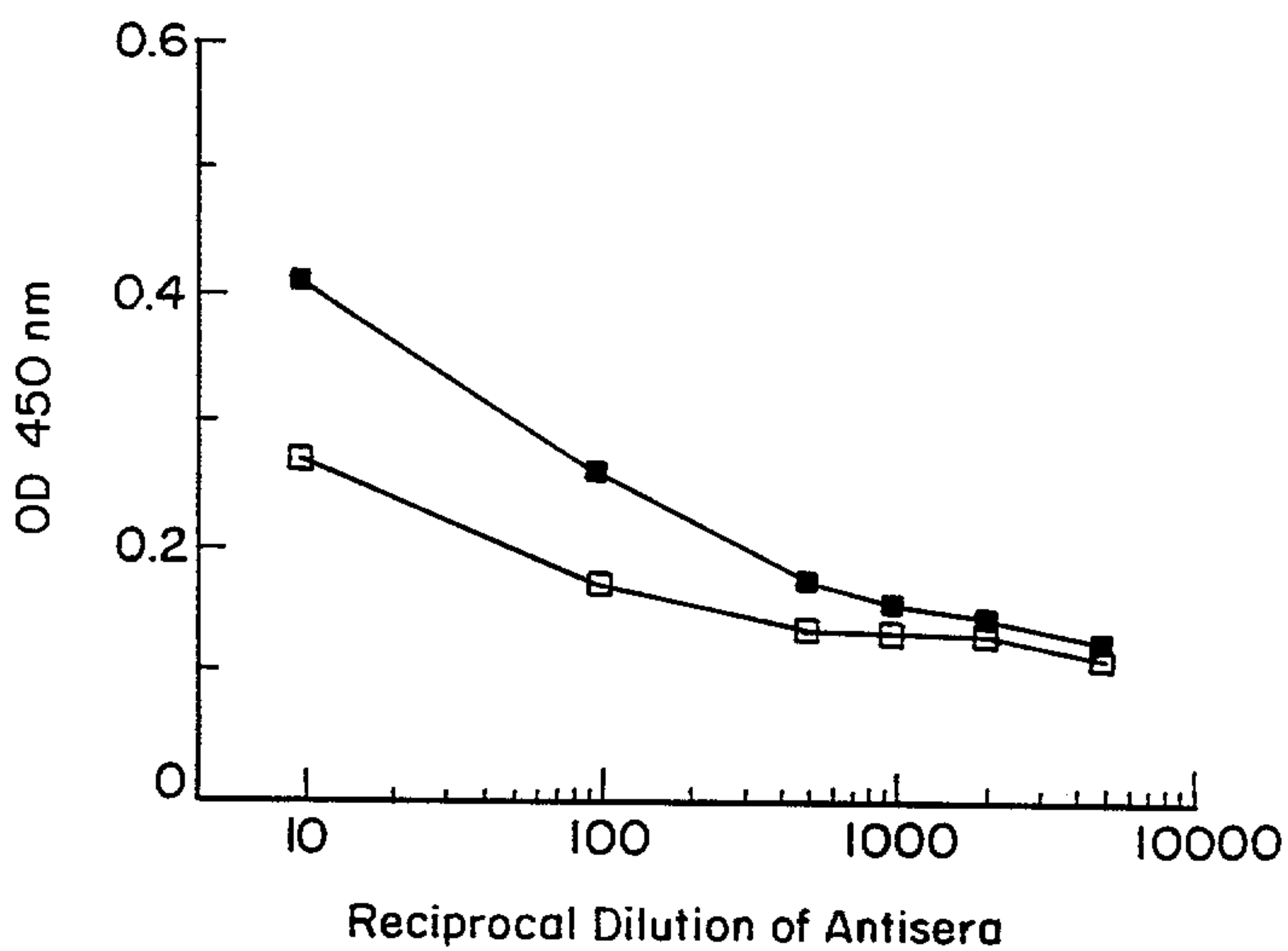


FIG. 12

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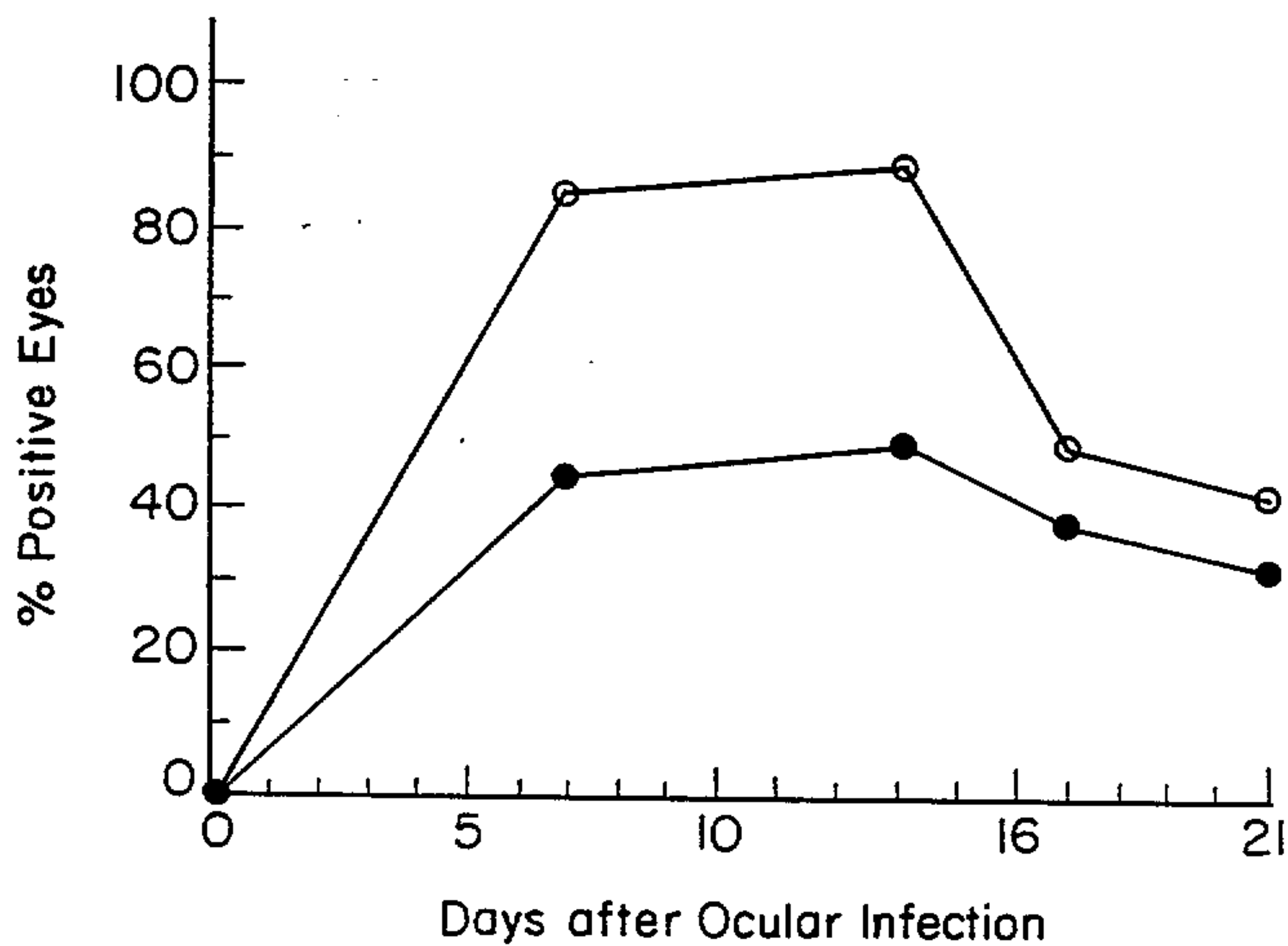


FIG. 13

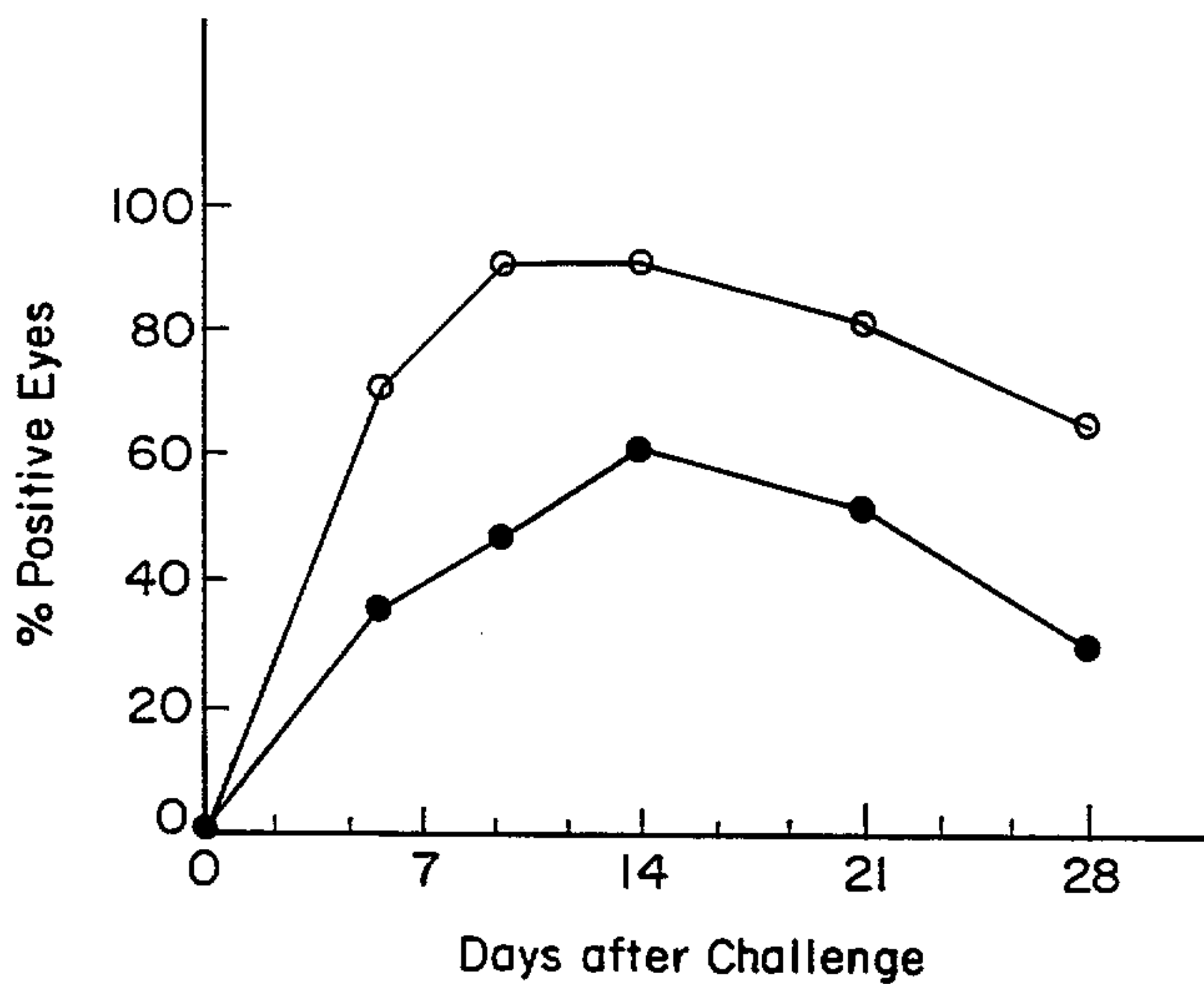


FIG. 14

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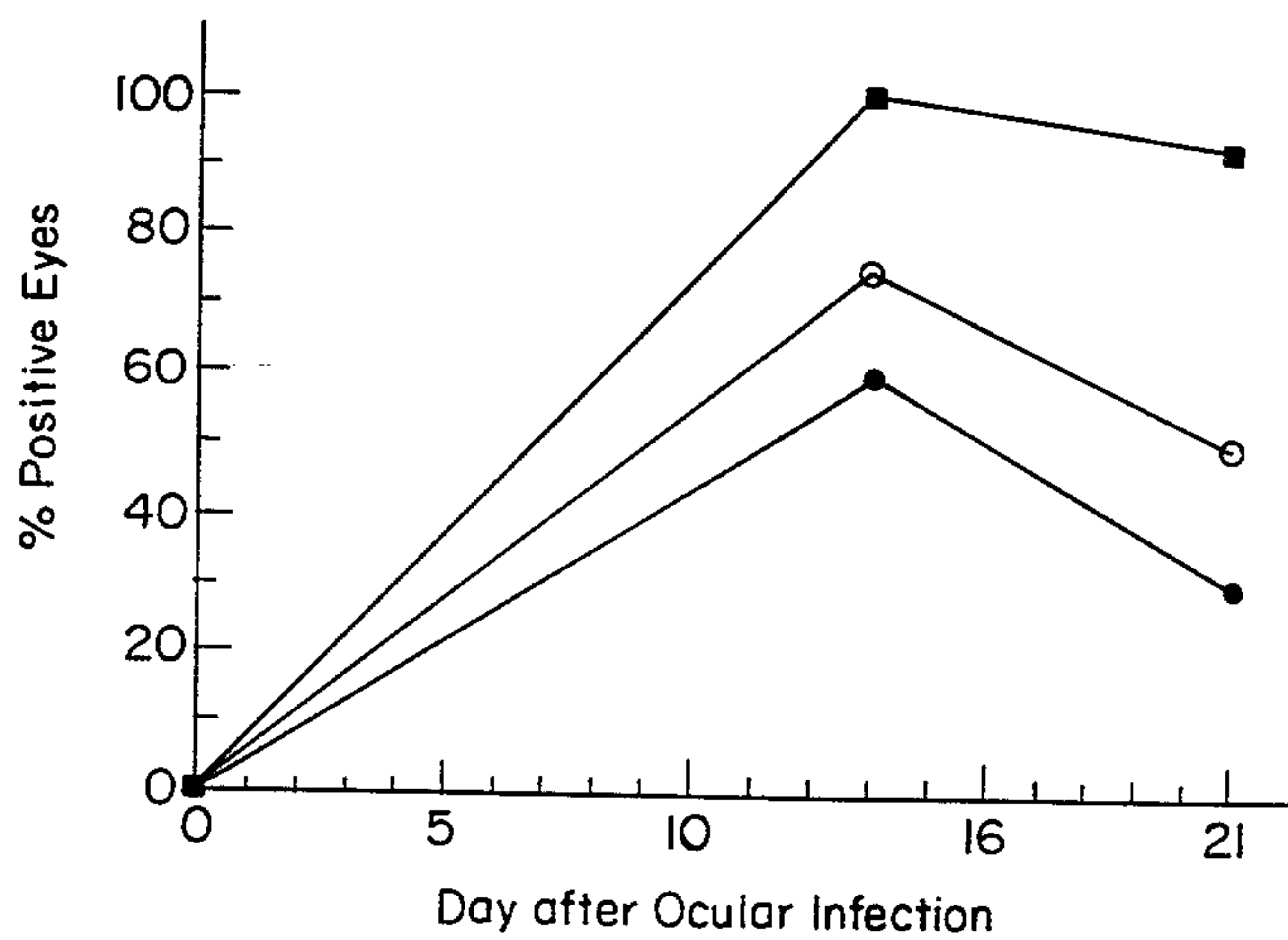


FIG. 15

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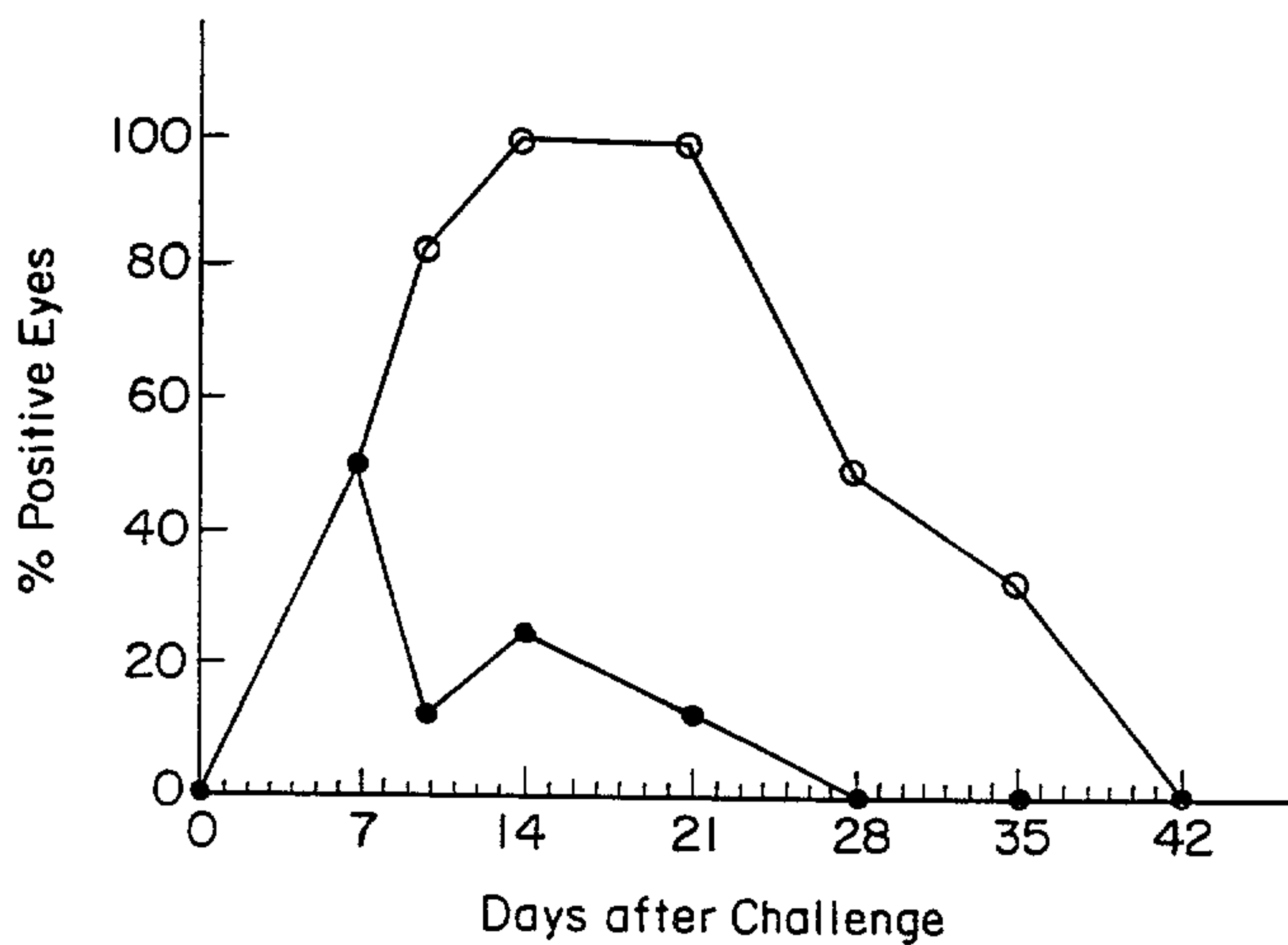


FIG. 16(A)

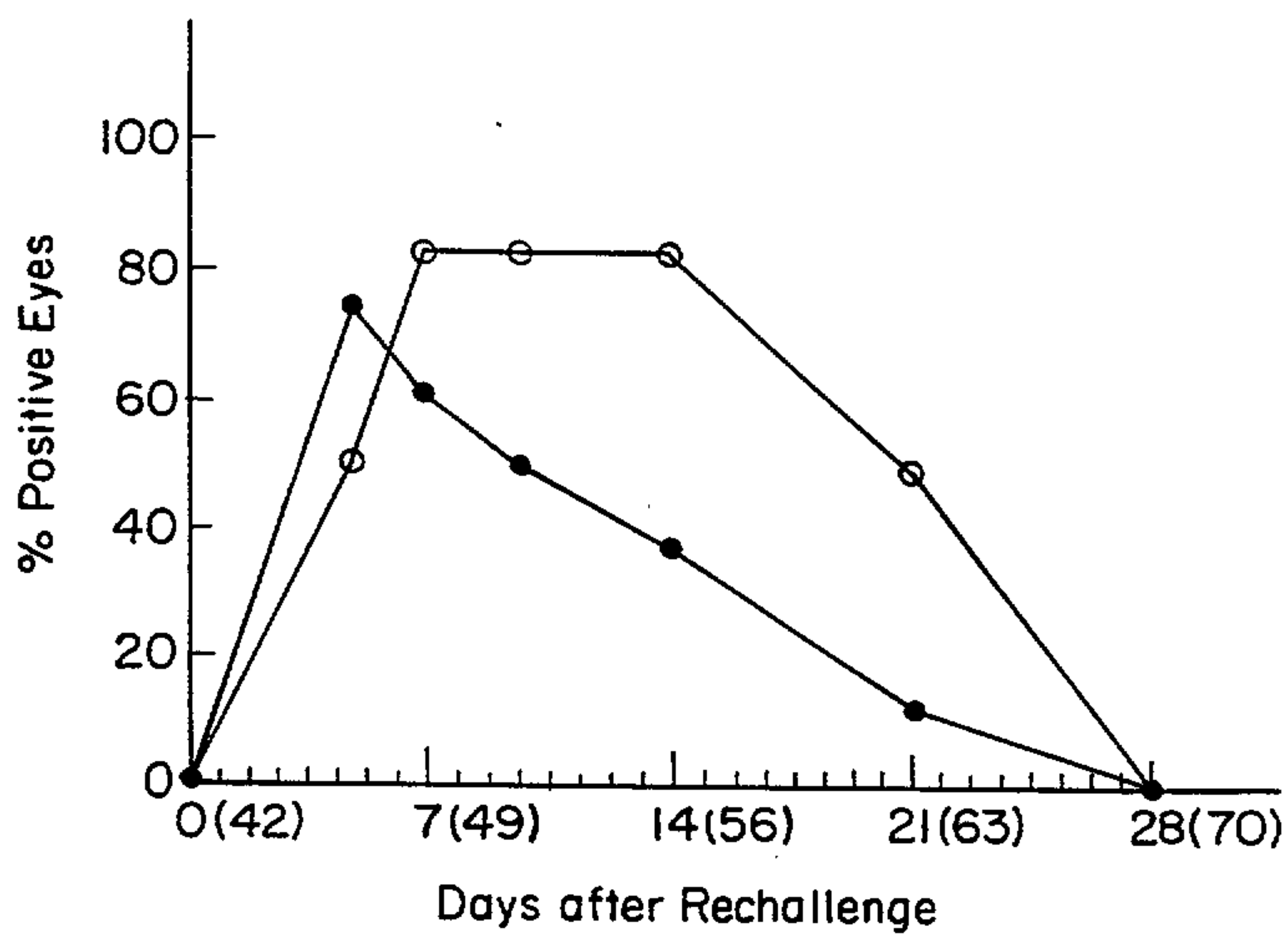
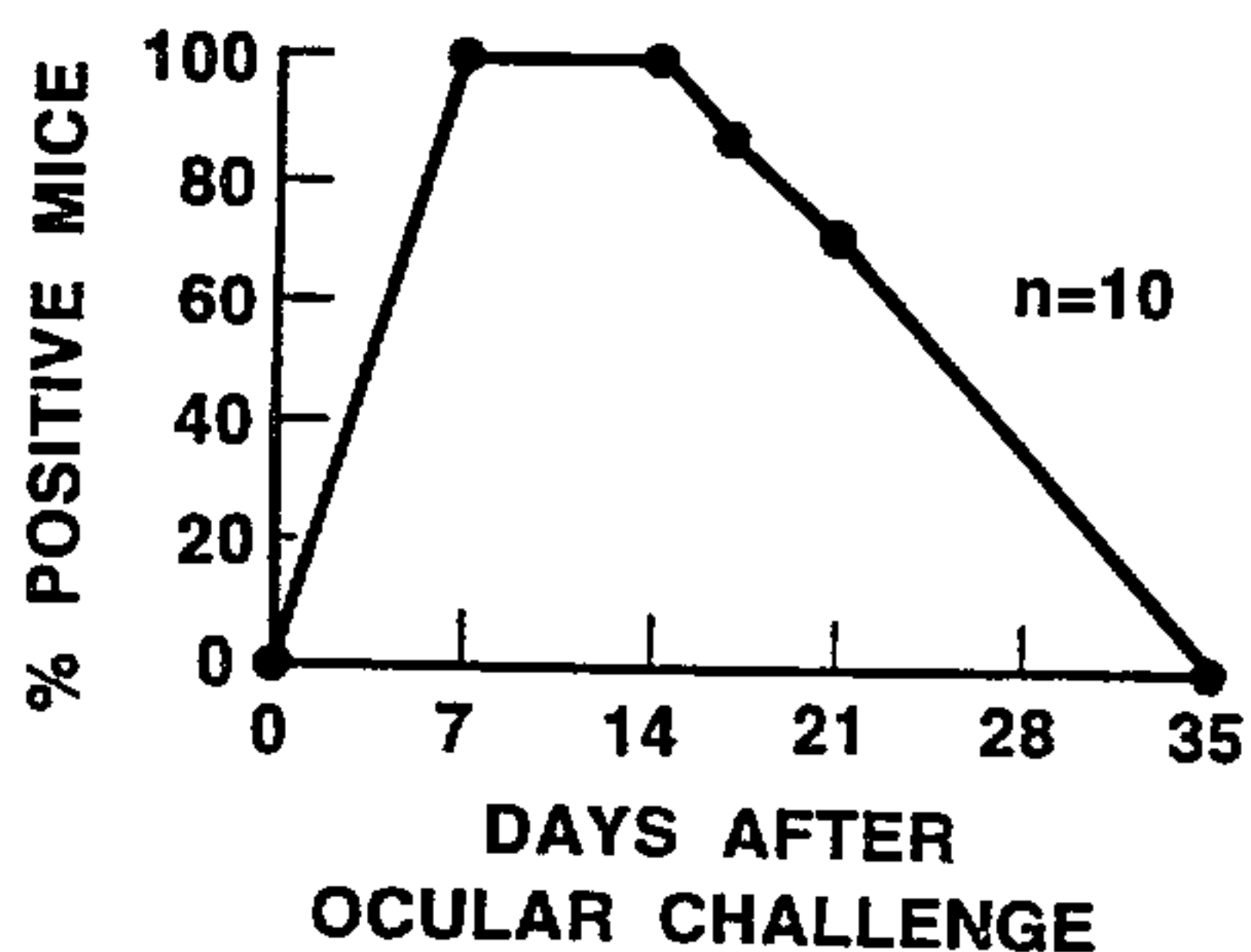


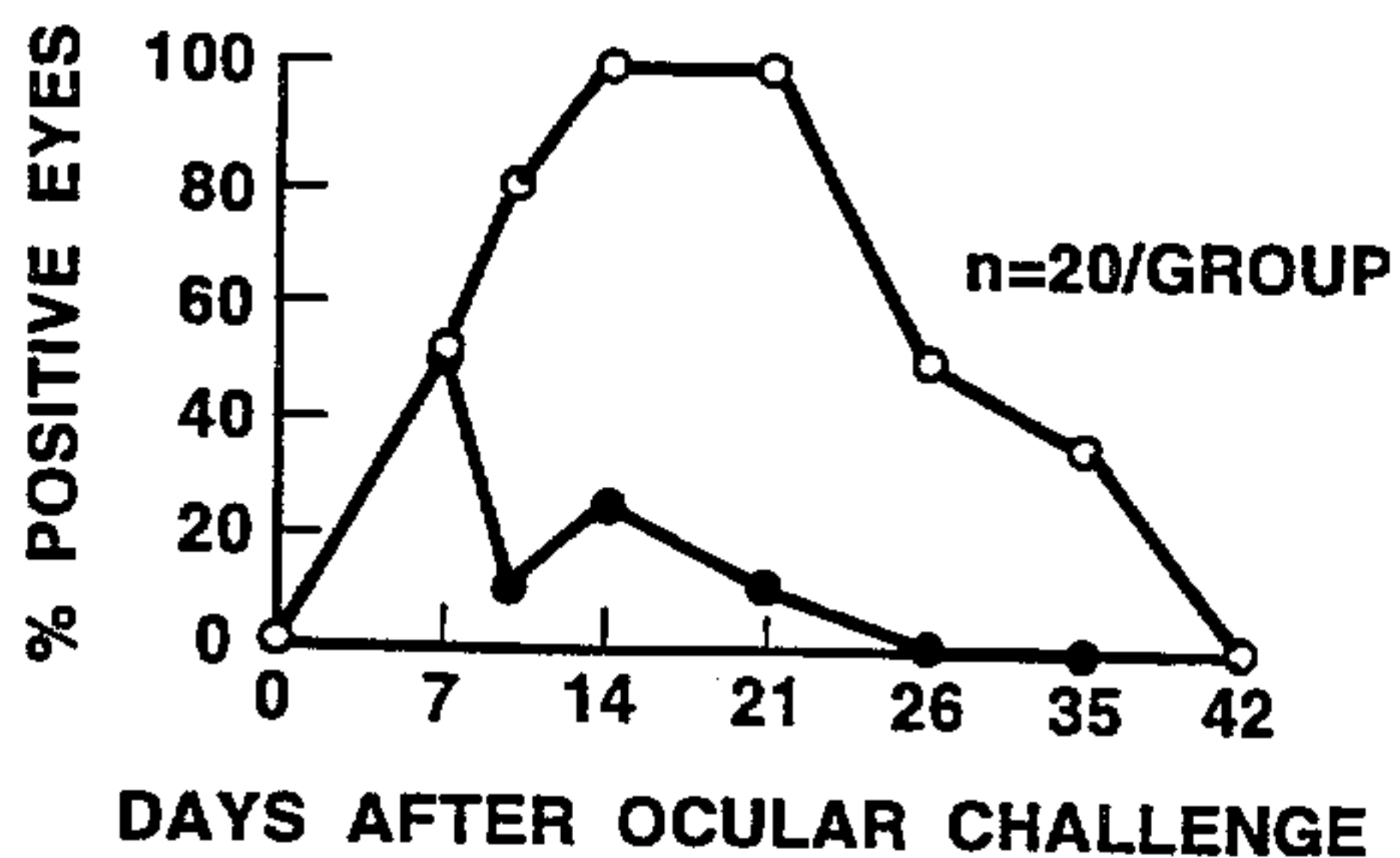
FIG. 16(B)

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OCULAR INFECTION IN BALB/c MICE

**Fig. 17**

ANTI-Id PROTECTS AGAINST OCULAR INFECTION

**Fig. 18**

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