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(57) Abstract

These are pharmaceutical compounds used to treat or prevent diseases caused by the over-production of a specific wild-type cytokine, characterised by the fact that they contain as an active principle at least one mutant of that wild-type cytokine. In its widest scope the invention consists in the use of muteins of a specific wild-type cytokine, that are also receptor antagonists of the latter, as immunogens to elicit antibodies directed against this wild-type cytokine and capable of neutralising it in those diesases caused by its excessive production. Figure 4 shows that antibodies developed in NSE/hIL-6 transgenic mice immunised with a human interleukin 6 mutein are capable of recognising wild-type interleukin 6 and of neutralising its biological activity.

USE OF MUTEINS OF WILD-TYPE CYTOKINES AS IMMUNOGENS

DESCRIPTION

The present invention relates to the use of muteins of a specific wild-type cytokine, that are also receptor antagonists of the latter, as immunogens to elicit antibodies against the wild-type cytokine, said antibodies being capable of neutralising the biological activity of the wild-type cytokine in diseases caused by an excessive production of the latter.

It is a known fact, for example, that human interleukin 6 (hIL-6) is a polypeptide of 184 aminoacids belonging to the class of helical cytokines. Interleukin 6 is a multi-functional cytokine produced by various cell types. It acts as a differentiation and growth factor on cells of various types, for example the cells of the immune system, hepatocytes, kidney cells, haematopoietic stem cells, keratinocytes and neurons. However, excessive production of hIL-6 causes a number of diseases, such as chronic auto-immune disorders, systemic lupus erythematosus, myeloma/plasmacytoma, post-menopausal osteoporosis and cancer cachexy.

There is thus a need in this specific sector to counteract the excessive production of a wild-type cytokine in general, and of hIL-6 in particular, both in terms of prevention and of cure.

The use of the present invention enables this need to be satisfied, offering at the same time other advantages which will become clear from the following.

A subject of the present invention is the use of mutants of human interleukin 6 for the preparation of pharmaceutical compositions for the treatment or prevention of diseases caused by an over-production of this specific wild-type cytokine.

A further subject of the present invention are use of mutein of human interleukin 6 to treat or prevent diseases caused by overproduction of human interleukin 6, or vaccines for the prevention of said diseases containing as an active principle at least one mutant of human interleukin 6.

The pharmaceutical compositions according to the present invention may be formulated according to known methods, which requires for example the presence of a pharmaceutically acceptable vehicle. Examples of these vehicles and methods of formulation can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable compound suitable for effective administration, these compounds must contain an effective amount of the active principle according to the present invention. The pharmaceutical compounds of the present invention are administered to an individual in amounts adequate to the disease, the weight, the sex and the age of the individual in question. Other factors include the method of administration. The pharmaceutical compounds according to the invention may be administered in a wide range of manners, for example subcutaneously, topically, orally and by intramuscular injection.

The pharmaceutical compositions according to the present invention, containing as an active ingredient at least one mutein of a wild-type cytokine, may be administered at therapeutic doses in a wide variety of forms, in conventional administration vehicles.

For example, they can be administered in doses to be taken orally in the form of tablets, capsules, pills, powders, granules, elixirs, ointments, solutions, suspensions, syrups and emulsions, or by injection. In the same way, these

compounds according to the invention may be administered intravenously, intraperitoneally, subcutaneously, topically with or without occlusion, or intramuscularly. All the above forms of dosage are well known to those skilled in the pharmaceutical field. In any case an effective, but non toxic amount of the active principle according to the invention must be used.

The daily dose of this active principle may vary within a wide range of between 0.01 to 1000 mg per adult/per day. An effective amount of the active principle according to the invention is usually provided at a dosage of between approximately 0.001 mg/kg and approximately 100 mg/kg of body weight per day.

According to the present invention, the active principle, which is made up of muteins of a specific wild-type cytokine, may be administered typically in a mixture with suitable diluents, excipients or pharmaceutical vehicles (generally referred to by the general term "vehicles") suitably selected so as to bear in mind the form of administration desired.

The preparation of vaccines according to the present invention is known to persons skilled in this field. Typically, these vaccines are prepared in an injectable form, either as solutions or as suspensions. The preparation can be emulsionated, or the active principle can be incapsulated in lyposomes. The active immunogenic ingredient is often mixed with pharmaceutically acceptable excipients that are compatible with the active ingredient. Suitable excipients are, for example, water, dextrose, glycerol, ethanol or the like and combinations thereof. Furthermore, if desired, the vaccine may contain small amounts of additional substances, such as, for example, wetting or emulsionating agents, pH

buffering agents, and/or adjuvants to increase the effectiveness of the vaccine.

The vaccines according to the invention are preferably administered parenterally, for example by means of either intramuscular or subcutaneous injection. Other formulations suitable for other methods of administration include suppositories and oral formulations. These compounds contain from 10 to 95% of active ingredient, preferably between 25 and 70%.

According to one aspect of the present invention, there is provided use of a mutein of human interleukin-6 that is also a receptor antagonist of human interleukin-6, for treating or preventing diseases caused by overproduction of human interleukin-6, wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6.

According to another aspect of the present invention, there is provided use of a mutein of human interleukin-6, that is also a receptor antagonist of human interleukin-6, for the preparation of a pharmaceutical composition comprising said mutein as immunogen wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and wherein said mutein is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6, said pharmaceutical composition being suitable for the treatment of prevention of diseases caused by over-production of human interleukin-6 by vaccination.

According to still another aspect of the present invention, there is provided a vaccine for immunization

against human interleukin-6, over-production of which causes a disease, containing an active immunogenic principal at least one mutein of human interleukin-6 that is also a receptor antagonist of human interleukin-6, wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and wherein said mutein is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6, in a pharmaceutically effective amount in a diluant, excipient or pharmaceutically acceptable vehicle.

According to yet another aspect of the present invention, there is provided a pharmaceutical composition comprising a mutein of human interleukin-6 that is also a receptor antagonist of human interleukin-6, and a pharmaceutically acceptable diluent, for use in treating or preventing a pathology caused by or related to over-production of interleukin-6, wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6, and wherein said pathology is selected from the group consisting of chronic auto-immune disorders, systemic lupus erythematosus, myeloma/plasmacytoma, post-menopausal osteoporosis and cancer cachexy when said diseases are caused by an over-production of interleukin-6.

Up to this point a general description has been given of the present invention. With the aid of the following examples a more detailed description will now be given, indicating specific situations that can be referred to the present invention, and aimed at giving a clearer understanding of the aims, characteristics, advantages and possible applications thereof.

Figures 1a and 1b show the results of the experiment of immunisation of normal and NSE/hIL-6 transgenic mice with wild-type hIL-6.

Figures 2a and 2b show the results of the experiment of immunisation with the mutant form, briefly indicated as Sant1 (containing the mutations Tyr 31 Asp, Gly 35 Phe, Ser 118 Arg, Val 121 Asp, Gln 175 Ile, Ser 176 Arg, Gln 183 Ala and being an antagonist of hIL-6) in normal and in NSE/hIL-6 transgenic mice.

Figure 3 shows the results of the experiment aimed at verifying whether or not the antobodies developed in mice against Sant1 are also capable of recognising wild-type IL-6.

Figure 4 shows the results of the experiment aimed at verifying whether or not the antibodies developed in NSE/hIL-6 transgenic mice immunised with the wild-type interleukin 6 mutant Sant1 are capable of neutralising the biological activity of wild-type human interleukin 6.

Figures 5a and 5b show the results of experiments carried out in example 7.

Figures 6a and 6b show the results of experiments carried out in example 11.

EXAMPLE 1

Immunisation of NSE/hIL-6 transgenic mice, with immune tollerance for hIL-6, using hIL-6 and Sant1

The NSE/hIL-6 transgenic mice have integrated into their genome the cDNA of human interleukin 6, under the control of the rat neuro-specific enolase gene promote.

The theory of tollerance was verified by attempting to immunize the above mentioned NSE/hIL-6 transgenic mice (a group of five mice) with recombinant human IL-6; as a control, siblings born in the same litter, but without the transgene were used. A group of five NSE/hIL-6 transgenic mice was also immunised with a mutant form of IL-6, called Sant1 (which does not have residual biological activity on human cells and behaves as an h-IL 6 receptor antagonist), which contained the seven mutations indicated below: Tyr 31 Asp, Gly 35 Phe, Ser 118 Arg, Val 121 Asp, Gln 175 Ile, Ser 176 Arg, Gln 183 Ala. In this case also five siblings from the same litter but without the transgene were used as controls.

The immunisation protocol was as follows: at time zero a sample of blood (pre-immune sample) was taken in sequence from each animal, both transgenic and non, and then each animal was immunised intraperitoneally (IP) with 100 µg of antigen (wt hIL-6 or Sant1, according to the group of mice) in the presence of Complete Freund Adjuvant (CFA). Ten days after the first immunization, a blood sample was taken (sample 1). Twenty days after first immunization a second immunization was carried out (first booster), again using 100 µg of antigen, in the presence of Incomplete Freund's Adjuvant (IFA), followed ten days later (30 days from first immunization) by a second blood sample (sample 2). Finally, fourty days after the first immunization a third immunization was carried out (second booster) again using 100 µg of antigen, in the presence of IFA, followed ten days later (50 days from first immunization) by the third blood sample (sample 3). The

corresponding serum was prepared from each of the blood samples, according to the state of the art.

At this point the "ELISA" method (Enzyme Linked ImmunoSorbent Assay) was used to test whether or not antibodies directed against the antigen used for immunization were present in the serum obtained from the second and third samples. To do this, the same antigen used for immunization was bound in a non co-valent manner to the bottom of the wells in culture plates specially produced for this type of experiment (ELISA plates). Immobilisation of the antigen took place by incubating 100 µl of a solution of antigen dissolved at 10 µl/ml in 1X PBS for 14 hours at room temperature in each of the wells to be coated (an operation termed "coating"). After immobilisation of the antigen, the plastic in the wells was coated with proteins, incubating a solution of 0.8% BSA (Bovine Serum Albumin) in 1X PBS for 4 hours at room temperature in each of the wells (an operation termed "blocking"). After removal of the "blocking" solution, 100 µl of each serum, suitably diluted, were incubated singly in each well for 90 minutes at room temperature: during this stage, if there are in the serum any antibodies for the antigen immobilised on the bottom of the well, these will bind the antigen itself, and will in turn be bound to the bottom of the well. After the 90 minute incubation period, the serums were removed from the wells and, after adequate washing, 100 µl of 0.8% BSA in 1XPBS containing rabbit antibodies directed against mouse antibodies were added to each well, and incubation was continued for 50 minutes at room temperature. During this stage, if mouse antibodies have recognised and bound the antigen immobilised on the bottom of the well, these mouse antibodies will be recognised and bound by the rabbit antibodies, which will thus in turn be immobilised on the bottom of the well. Furthermore, the rabbit antibodies directed against mouse antibodies (used diluted at a ratio of 1:100 in PBS/BSA for the above

mentioned experiment), which are produced and distributed by the company DAKO, are covalently linked to an enzyme, horseradish peroxidase. After the 50 minutes of incubation, the solution containing rabbit antibodies was removed, and the wells were adequately washed. At this point, 100 µl of a solution containing a substrate (TMB: 3,-3',-5,-5'-tetramethyl-benzydine-dichloride) for horseradish peroxidase were added to each well. The enzyme converts the substrate into a product that absorbs visible light at 450 nanometers; thus the amount of conversion can be calculated by spectrophotometric measurement of the light absorbtion at 450 nm in each single well, using an ELISA reader. If the experiment is carried out correctly, the absorbance (that is to say the amount of light absorbed) is proportional to the amount of enzyme, which is in turn proportional to the amount of rabbit antibody, which is in turn proportional to the amount of mouse antibody directed against the antigen originally present in the serum. Thus, the measurement of the absorbance gives an estimate of the amount of mouse antibody directed against the antigen present in the serum. This is true if the mouse antibody against the antigen is the factor limiting the chain of reactions described above. Thus, to obtain the appropriate conditions, it is advisable to carry out a series of dilutions (from 1:33 to 1:8100) for each serum to be examined, so that, for each serum there will be certain dilutions in which the amount of mouse antibody for the antigen used for immunization will be sufficiently high to be measured with precision, but not so high as to saturate the system.

The results of the wild-type IL-6 (wtIL-6) immunization experiment are illustrated in figures 1a and 1b for a typical normal mouse and for a typical NSE/hIL-6 transgenic mouse. As can be seen from figure 1a, the normal mouse has developed a large amount of anti-wtIL-6 antibodies, so much so that it is possible to detect a

signal even when the serum is diluted to 1:8100. Vice versa, as can be seen from figure 1b, the transgenic mouse has developed a much lower amount of antibodies: in fact the signal ceases when the serum from the second sample is diluted at a ratio of 1:300 and when the serum from the third sample is diluted at a ratio of 1:2700. To carry out objective measurements that are comparable for all animals, the dilution of serum that gives a reading of 0.5 $O.D._{450}$ above the highest reading of the pre-immune serum from the same animal has been conventionally termed "titer". Figures 1a and 1b show how the titer of the second and third samples are calculated for normal mice and for the NSE/hIL-6 transgenic mice, respectively.

Figures 2a and 2b show the results of the experiment on immunisation using a mutant form of IL-6, Sant1, of a typical normal mouse and of a typical NSE-hIL-6 transgenic mouse, respectively. In this case both the mice developed a large amount of anti-Sant1 antibodies. In fact, it is possible to detect a relatively strong signal even when the serum is diluted to 1:8100. Figures 2a and 2b show how the titers of the second and third samples are calculated for the normal mouse and for the transgenic mouse.

Table 1 gives a summary of titer data (calculated as indicated above) for the second and third sample for all the mice innoculated during this experiment.

TABLE 1

IMMUNOGENICITY OF Sant 1

MICE GROUP	SERUM TITER 2nd sample	SERUM TITER 3rd sample	
WT/wtIL-6			
16	245	5150	
17	1100	3900	
18	1250	2600	
19	430	1600	
20	210	1800	
Average	674	3010	
NSE/wtIL-6			
11	80	92	
12	90	215	
13	24	155	
14	135	135	
15	27	54	
Average	71	130	Ratio of wt mice titer/ NSE mice titer
	9.5	**23**	
WT/Sant1			
6	150	270	
7	1450	dead	
8	2900	3600	
9	1300	2700	
10	3300	3800	
Average	1820	2600	
NSE/Sant1			
1	4000	4000	
2	21.5	49	
3	1750	>10000	
4	1750	1150	
5	2000	1800	
Average	1900	3400	
			Ratio of wt mice titer/
	1	**0.76**	

NSE mice
titer

As regards the mice innoculated with the wtIL-6 antigen, it can be seen that, apart from the variations between one animal and another, on average the non-transgenic mice developed an antibody response against wtIL-6 10-20 times stronger than that obtained in the NSE/IL-6 transgenic mice, this being proof of the fact that the transgenic mice have developed an immune tollerance of human hIL-6.

On the contrary, as regards the mice innoculated with the antigen Sant 1, it can be seen that, once again apart from the variations between one animal and another, on average the non-transgenic mice developed an antibody response - equivalent to that obtained in NSE/hIL-6 transgenic mice, suggesting that the seven mutations, which when introduced into hIL-6 generated Sant1, have also rendered the mutant Sant1 a completely foreign protein for an immune system that otherwise displays tollerance to hIL-6.

EXAMPLE 2

The antibodies developed in the serum of NSE/hIL-6 immunized with Sant1 are capable of recognising not only Sant1, but also wild-type hIL-6

This was to test whether or not the antibodies developed by mice against Sant1 were capable of recognising not only Sant1 itself, but also wild-type hIL-6 (wt hIL-6). This hypothesis was tested once again using an ELISA test. After having verified the fact that antibodies developed in NSE/hIL-6 transgenic mice immunised with Sant1 are capable of recognising and binding the same mutant Sant1 immobilised on the bottom of ELISA plate wells, in a second experiment the wild-type IL-6 was bound in a non-covalent manner to the bottom of ELISA plate wells. After the phase in which the plastic of the wells is saturated with BSA, dilutions

of serum from mice immunized with Sant1 were added to the wells to verify whether or not there was a presence of antibodies capable of recognising wtIL-6. For example, figure 3 shows the results of the experiment for the NSE/hIL-6 transgenic mouse. As can be seen, the antibody responses obtained for Sant1 and for wild-type IL-6 are extremely similar. In other words, it is possible to detect the presence of antibodies that have bound the wtIL-6 immobilised on the bottom of the wells in the serum of the third sample, even when the latter is diluted to a ratio of 1:8100. It should be noted that when the NSE/hIL-6 transgenic mice are immunised directly using wild-type IL-6, the antibody response directed at wild-type IL-6 itself is much lower: in effect it is not possible to detect anti-wtIL-6 antibodies in serum diluted to 1:8100 (see figure 1). Other NSE/hIL-6 transgenic mice showed a response similar to that of the mouse illustrated in the example.

EXAMPLE 3

The antibodies developed in NSE/hIL-6 immunised with Sant1 are also capable of neutralising the biological activity of wild-type hIL-6

The object was to test whether or not these antibodies, developed in NSE/hIL-6 transgenic mice immunised with Sant1, but capable of recognising wild-type IL-6, are also capable of neutralising the biological activitiy of wild-type IL-6 itself.

The biological activity of wild-type IL-6 under consideration was the ability to stimulate transcription by the C-reactive protein gene promoter in human Hep3B hepatoma cells. The effectiveness of stimulation of transcription was measured according to the state of the art (Gregory, B., Savino, R. and Ciliberto, G., J. Immunological Methods, 170, 47-56, 1994). Human Hep3B hepatoma cells were stimulated with 4 ng/ml of wild-type IL-6, in the presence of serial dilutions of the serum obtained in the third sample from two NSE/hIL-6

transgenic mice #4 and #5 (both immunised with Sant1). The results of the experiment are given in figure 4. It can be seen that both the serums diluted to 1:100 almost completely inhibit the biological activity of wild-type hIL-6 at 4 ng/ml on human hepatoma cells.

At this point, investigations were carried out as to whether or not the occurance of this cross-reactive antibody response against wild-type hIL-6 was capable of altering the levels of wild-type hIL-6 measured in the serum of the NSE/hIL-6 transgenic mice immunized with Sant1. The hIL-6 levels were measured according to the state of the art by means of a "sandwich" ELISA test, using a commercially available kit produced by the company "R&D Systems", and scrupulously following the manufacturer's instructions. The levels of hIL-6 found both in the pre-immune sample and in the third sample were measured for four transgenic mice who had been immunized with Sant1. The results are summarised in table 2. As can be seen, immunisation using Sant1, as well as causing the appearance of a strong antibody response that recognises both Sant1 itself and the wtIL-6, also causes an average decrease of over 500 times in the levels of hIL-6 that can be detected in the serum of the NSE/hIL-6 mice using the ELISA "sandwich" kit produced by R&D Systems.

Table 2 - Decrease in the levels of hIL-6 that can be detected in the serum of NSE/hIL-6 mice

	hIL-6 levels in the serum	
	Pre-immune serum	3rd sample
Mouse No. 1	26 ng/ml	0.02 ng/ml
Mouse No. 2	39 ng/ml	0.01 ng/ml
Mouse No. 3	28 ng/ml	0.10 ng/ml
Mouse No. 4	35 ng/ml	0.10 ng/ml
average	32 ng/ml	0.057 ng/ml

EXAMPLE 4

The antibodies developed in NSE/hIL-6 mice vaccinated with Sant1 are able to neutralize the biological activity of wild-type hIL-6 also *in vivo*

It is well known that IL-6 induces the production of a series of proteins (called "acute phase proteins") by the liver. Serum Amyloid A, hereon referred as SAA, shows intense and rapid increase during acute events.

The objective was to test whether or not these antibodies, developed in NSE/hIL-6 transgenic mice vaccinated with Sant1, and capable of cross-reacting with wtIL-6, are also capable of neutralising the biological activity of wild type hIL-6 *in vivo*, measured as inhibition of the increase of mouse SAA (mSAA) following the injection of hIL-6. To this purpose, a blood sample (hereon referred as pre-injection sample) was taken from unimmunised (control) NSE/hIL-6 mice, from wt hIL-6 immunised and from Sant1 immunised NSE/hIL-6 mice. After the animals recovered from the bleeding, they were injected intra-peritoneally with 10 μg of wt hIL-6. Nine hours after the injection, a second blood sample (hereon referred as post-injection sample) was taken from both groups of animals. The mSAA levels were measured both in the pre-injection sample and in the post-injection sample according to the state of the art by means of a "sandwich" ELISA test, using a commercially available kit produced by the company "Biosource International", and scrupulously following the manufacturer's instructions. The results are summarised in Table 3. As can be seen, apart from the variations from between one animal and another, on average in unimmunized mice injection of 10 μg of hIL-6 determined a significant increase in the serum SAA levels, increase which was absent in the Sant1 vaccinated mice injected with the same amount of hIL-6. Therefore, vaccination with Sant1, as well as causing the appearance of a strong antibody response that recognises both Sant1 itself and the wthIL-6 and that is able to

neutralize wt hIL-6 biological activity *in vitro* on human hepatoma cells, prevents *in vivo* the increase of mSAA levels induced by injection of hIL-6, in other words in neutralizes the biological activity of hIL-6 also *in vivo*. It should be noticed that immunization with wild type hIL-6, induces the production of a low amount of anti hIL-6 antibodies (see Example 1), and this is not not able to prevent *in vivo* the increase of mSAA levels induced by injection of hIL-6, because mice immunized with wild type hIL-6 show an increase in the serum SAA levels comparable to the one observed in unimminised control mice.

Table 3 - Increase in the levels of mSAA detectable in the serum of Sant1-immunised and control NSE/hIL-6 transgenic mice after hIL-6 injection.

		mSAA levels in the serum		mSAA levels fold increase	
Mice group	mouse number	pre-inject. sample	post-inject. sample	single mouse	group average
	1	68 µg/ml	- 24 µg/ml	0.35	
Sant1	3	23 µg/ml	14 µg/ml	0.61	
immunised	28	83 µg/ml	93 µg/ml	1.12	1.13
	36	62 µg/ml	105 µg/ml	1.7	
	37	95 µg/ml	177 µg/ml	1.87	
	5	112 µg/ml	718 µg/ml	6.4	
	8	41 µg/ml	694 µg/ml	16.9	
Control	14	140 µg/ml	613 µg/ml	4.4	9.01
unimmunised	21	47 µg/ml	479 µg/ml	10.13	
	22	51 µg/ml	412 µg/ml	8.1	
	51	53 µg/ml	433 µg/ml	8.15	
	11	72 µg/ml	784 µg/ml	10.3	
wt hIL-6	12	54 µg/ml	380 µg/ml	6.9	8.9
immunised	13	28 µg/ml	310 µg/ml	11.1	
	15	16 µg/ml	117 µg/ml	7.2	

EXAMPLE 5

Immunisation of NSEhIL-6 transgenic mice with hIL-6 and Sant1 formulated in a different adjuvant, aluminum hydroxide

It is well known that different antigens behaves differently when formulated in different adjuvants (Gupta, R. K. and Siber, G. R., Vaccine, 13, 1263-1276, 1995). The Complete (or Incomplete) Freund Adjuvant used in the immunization experiment described in the Example 1 cannot be used in humans because of side effects, mostly local reactions at the site of injection such as granuloma and cyst formation (Gupta, R. K. and Siber, G. R., Vaccine, 13, 1263-1276, 1995). The objective was to determine whether a similar immune response, with high titer antibodies against Sant1 and also against wt hIL-6 could be raised in NSE/hIL-6 transgenic mice using an adjuvant commonly used for vaccination in humans. For this purpose, aluminun hydroxide was chosen, because it is today the common adjuvant for human use, with an excellent track record of safety (Gupta, R. K. and Siber, G. R., Vaccine, 13, 1263-1276, 1995).

Groups of 8-10 NSE/hIL-6 transgenic mice (plus ten non-transgenic siblings born in the same litters used as controls) were immunized intraperitoneally with 100 μg of antigen (either Sant1 or wild type hIL-6) formulated in aluminum hydroxide at 1 mg/ml in a total volume of 100 μl (100 μg of aluminum hydroxide) for each injection, using an immunization protocol identical to the one described in the Example 1. The second blood sample (taken after the second injection or first booster) and the third blood sample (taken after the third injection or second booster) were then tested for the presence of antibodies against the antigen used for the immunization by means of the "ELISA" described in the Example 1.

To carry out objective measurements that are comparable for all animals the dilution of serum that

gives a reading of 0.5 O.D.$_{450}$ above the highest reading of the preimmune serum from the same animal has been conventionally termed "titer". The titer was measured as illustrated in figures 1a, 1b, 2a and 2b for both normal and transgenic mice immunized with wild type hIL-6 and with Sant1. The results are reported in Table 4.

In general the amount of antibodies against the antigen obtained in this immunization experiment (titer) is higher as compared with the amount of antibodies obtained in the immunization experiment described in Example 1; indeed, it is part of the state of the art the fact that aluminum adjuvants are the adjuvants of choice for induction of serum antibodies (Gupta, R. K. and Siber, G. R., Vaccine, **13**, 1263-1276, 1995). More in particular, as regards the mice imunized with the wt hIL-6 antigen, it can be seen again that (apart from the variation from one animal and another) on average the non-transgenic mice developed an antibody response against wt hIL-6 12-18 times stronger than that obtained in NSE/hIL-6 transgenic mice, this being proof of the fact that the transgenic mice have developed an immune tolerance to human IL-6 also when this antigen is injected formulated in aluminum hydroxide as adjuvant. On the contrary, as regards the mice immunized with Sant1 formulated in aluminum hydroxide, it can be seen that also in this case on average the non-transgenic mice developed an antibody response equivalent to that obtained in NSE/hIL-6 transgenic mice, as it was in the immunization experiment described in Example 1, suggesting that the seven mutations (which when introduced into hIL-6 generated Sant1) have rendered the mutant a completely foreign protein also when formulated in aluminum hydroxide.

Table 4. Immunogenicity of Sant1 and of wt hIL-6 formulated in aluminum hydroxide and injected intraperitoneally and NSE/hIL-6 transgenic and wild type control mice.

Mice group	mouse number	SERUM TITER 2nd sample	SERUM TITER 3rd sample
Non-transgenic	30	21960	11848
mice	32	6070	15790
immunised	34	7460	46000
with	46	14145	19600
wild type	49	6240	18600
hIL-6 in	54	1270	6960
aluminum	56	218	320
hydroxide	58	5470	5260
via	63	2138	2373
I.P.	65	1200	3810
Averages of the group		**6617**	**13056**
NSE/hIL-6	35	450	530
mice	51	2200	1630
immunised	53	169	100
with	60	160	630
wild type	75	55	217
hIL-6 in	85	81	1440
aluminum	87	110	176
hydroxide	90	64	390
via	93	1900	1700
I.P.	99	194	555
Averages of the group		**538**	**737**

		20	7270	28185
	Non-transgenic	21	7580	30200
	mice	26	3555	54330
	immunised	27	2185	10960
	with	28	9060	35700
	Sant1 in	29	7910	34515
	aluminum	33	7380	43980
	hydroxide	34	1160	10550
	via I.P.	36	5785	19880
		43	5035	23155
	Averages of the group		**5692**	**29145**
	NSE/hIL-6	22	4520	33105
	mice	35	3516	28690
	immunised	38	9990	44680
	with	44	3580	14430
	Sant1 in	46	1480	9940
	aluminun	59	9780	20525
	hydroxide	61	2360	63970
	via I.P.	79	2250	26330
	Averages of the group		**4400**	**30210**

EXAMPLE 6

The antibodies developed in the serum of NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide are capable of recognising not only Sant1 but also wild-type hIL-6

This was to test whether the antibodies developed against Sant1 in NSE mice vaccinated with Sant1 formulated in aluminum hydroxide were capable of cross-reacting with wild type hIL-6 (wt hIL-6). This was tested once again by "ELISA" using the same methodology described in the Example 2. The antibody titer was calculated as previously described and as illustrated in

figures 1a, 1b, 2a and 2b, and the data obtained are reported in Table 5.

Table 5 - Cross-reactivity against wt hIL-6 of sera of NSE/hIL-6 transgenic mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intraperitoneally.

Mice group	mouse number	2nd sample serum titer wt hIL-6	2nd sample serum titer Sant1	3rd sample serum titer wt hIL-6	3rd sample serum titer Sant1
NSE/hIL-6	22	2525	4520	31180	33105
mice	35	1925	3516	11860	28690
vaccinated	38	7065	9990	43850	44680
with	44	3580	3580	10585	14430
Sant1 in	46	1480	1480	8675	9940
aluminun	59	9990	9780	20360	20525
hydroxide	61	2800	2360	52000	63970
via I.P.	79	1980	2250	14890	26330
Averages of the group		**3630**	**4400**	**22800**	**28750**

Again, also when aluminum hydroxide is used as adjuvant for the immunization, the antibody titers against Sant1 and wild type hIL-6 are similar. Again, it should be noticed that when the NSE/hIL-6 transgenic mice are immunised with wild type hIL-6, the antibody response directed against wild type hIL-6 itself is much lower: for instance in the third blood sample the average titer against wt hIL-6 is 737 in the group of NSE/hIL-6 mice immunised with wt hIL-6 (see Example 5) as compared with an average titer against wt hIL-6 of 22800 (13 times higher) in the group of NSE/hIL-6 mice vaccinated with Sant1.

EXAMPLE 7

The antibodies developed in NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide are also able to neutralise the biological activity of wild type hIL-6

The objective was to test whether or not these antibodies, developed in NSE/hIL-6 transgenic mice vaccinated with Sant1 formulated in aluminum hydroxide, but capable of recognising wild type hIL-6, are also capable of neutralising the biological activity of wild type hIL-6 itself.

The biological activity of wild-type IL-6 under consideration was the ability to stimulate transcription by the C-reactive gene promoter in human Hep3B hepatoma cells. The effectiveness of transcriptional stimulation was measured according to the state of the art (Gregory, B., Savino, R. and Ciliberto, G., *J. Immunological Methods*, **170**, 47-56, 1994). Human Hep3B hepatoma cells were stimulated with 4 ng/ml of wild-type hIL-6, and this extent of stimulation was taken as 100%, or with 4 ng/ml of wild type hIL-6 in the presence of serial dilutions of the serum obtained from the third blood sample from NSE/hIL-6 mice immunised with both Sant1 and wild type hIL-6; in the latter cases the extent of transcriptional stimulation was expressed as percent of the stimulation obtained in cells incubated with 4 ng/ml of wild type hIL-6 only. The results of the experiment are given in Figures 5a and 5b. It can be seen that the serum of all mice diluted 1:400 almost completely inhibits the biological activity of wild type hIL-6 at 4 ng/ml on human hepatoma cells. Therefore, the ability to neutralize the bioactivity of exogenously added hIL-6 on human hepatoma cells was even higher for the sera of animals immunized with aluminum hydroxide than in the case of animals immunized with CFA (see Example 3 and compare Figure 5a with Figure 4). When NSE/hIL-6 transgenic mice are immunised with wild type hIL-6 the

very low amount of anti hIL-6 antibodies obtained is not sufficient to inhibit wild type hIL-6 biological activity on human hepatoma cells (see Figure 5b).

Also in this case, investigations were carried out as to whether or not the occurrance of this cross-reactive immune response against wild type hIL-6 was capable of altering the levels of wild type hIL-6 measured in the serum of NSE/hIL-6 transgenic mice immunised with both Sant1 and wild type hIL-6. The hIL-6 levels were measured as described in the Example 3 both in the pre-immune sample and in the third sample of both groups of mice. The results are summarised in Table 6. As can be seen, vaccination using Sant1 formulated in aluminum hydroxide, as well as causing the appearance of a strong antibody response that recognises both Sant1 itself and the wild type hIL-6, also causes an average decrease of about 1,400 times in the levels of hIL-6 that can be detected in the serum of the NSE/hIL-6 transgenic mice. Immunisation using wild type hIL-6 itself formulated in the same adjuvant causes only a marginal decrease (3-fold as compared with 1,400-fold) in the levels of hIL-6 that can be detected in the serum.

Table 6 - Decrease in the levels of hIL-6 that can be detected in the serum of NSE/hIL-6 mice immunised with Sant1 and wt hIL-6 formulated in aluminum hydroxide.

Mice group	mouse number	hIL-6 levels in the serum (pg/ml) Pre-immune sample	3rd sample
NSE/hIL-6	35	27505 pg/ml	3522 pg/ml
mice	51	24950 pg/ml	2662 pg/ml
immunised	53	25817 pg/ml	4566 pg/ml
with	60	23283 pg/ml	9810 pg/ml
wild type	75	26373 pg/ml	8186 pg/ml
hIL-6 in	85	23000 pg/ml	8959 pg/ml
aluminum	87	25527 pg/ml	9172 pg/ml
hydroxide	90	25590 pg/ml	20041 pg/ml
via	93	23275 pg/ml	5834 pg/ml
I.P.	99	30830 pg/ml	6582 pg/ml
Averages of the group		**25585 pg/ml**	**7933 pg/ml**
NSE/hIL-6	22	27300 pg/ml	*9 pg/ml
mice	35	31778 pg/ml	*9 pg/ml
immunised	38	25223 pg/ml	*9 pg/ml
with	44	29385 pg/ml	41 pg/ml
Sant1 in	46	20600 pg/ml	*9 pg/ml
aluminum	59	22820 pg/ml	45 pg/ml
hydroxide	61	23125 pg/ml	*9 pg/ml
via I.P.	79	24510 pg/ml	*9 pg/ml
Averages of the group		**25593 pg/ml**	**18 pg/ml**

The star (*) indicates the lower limit of sensitivity of the assay.

EXAMPLE 8

The antibodies developed in NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide are able to neutralize the biological activity of wt hIL-6 also *in vivo*

The objective was to test whether or not these antibodies, developed in NSE/hIL-6 transgenic mice vaccinated with Sant1 formulated in aluminum hydroxide, but capable of recognising wtIL-6, are also capable of neutralising the biological activity of wild type hIL-6 *in vivo*, measured as the increase of mouse SAA (mSAA) serum levels induced in the mice by injection of hIL-6, as described in the Example 4. The experiment was performed as described in the Example 4 on unimmunised (control) NSE/hIL-6 mice and on NSE/hIL-6 mice immunized with Sant1 formulated in aluminum hydroxide. The results are summarised in Table 7. As can be seen, apart from the variations from between one animal and another, on average again in unimmunized mice injection of 10 μg of hIL-6 determined a 5- to 6-fold increase in the serum SAA levels. No increase was obtained in the mice vaccinated with Sant1 formulated in aluminum hydroxide, injected with the same amount of hIL-6.

Table 7 - Increase in the levels of mSAA detectable in the serum of Sant1-vaccinated and control NSE/hIL-6 transgenic mice after hIL-6 injection.

Mice group	mouse number	mSAA levels in the serum pre-inject. sample	mSAA levels in the serum post-inject. sample	mSAA levels fold increase single mouse	mSAA levels fold increase group average
Sant1 vaccinated	22	6.1 µg/ml	7.1 µg/ml	1.14	1.19
	35	4.4 µg/ml	5.5 µg/ml	1.24	
	38	7.4 µg/ml	9.9 µg/ml	1.34	
	44	4.6 µg/ml	6.5 µg/ml	1.4	
	46	5.2 µg/ml	6.9 µg/ml	1.33	
	59	6.7 µg/ml	7.5 µg/ml	1.13	
	61	8.6 µg/ml	10.2 µg/ml	1.19	
	79	8.8 µg/ml	6.8 µg/ml	0.77	
Control unimmunised	15	85 µg/ml	265 µg/ml	3.1	5.41
	16	99 µg/ml	366 µg/ml	3.7	
	19	296 µg/ml	2997 µg/ml	10.1	
	20	41 µg/ml	146 µg/ml	3.6	
	21 bis	45 µg/ml	276 µg/ml	6.1	
	55	41 µg/ml	303 µg/ml	7.4	
	56	75 µg/ml	291 µg/ml	3.9	

Therefore, vaccination with Sant1, as well as causing the appearance of a strong antibody response that recognises both Sant1 itself and the wthIL-6 and that is able to neutralize wt hIL-6 biological activity *in vitro* on human hepatoma cells, prevents *in vivo* the increase of mSAA levels induced by injection of hIL-6, in other words in neutralizes the biological activity of hIL-6 also *in vivo*.

EXAMPLE 9

Vaccination of NSEhIL-6 transgenic mice with hIL-6 and Sant1 formulated in aluminum hydroxide *via* intradermal administration route

Examples 5, 6, 7 and 8 above show that it is

possible to obtain in animals otherwise tolerant to wt hIL-6 a strong antibody response against wt hIL-6 itself, that is able to neutralize hIL-6 bioactivity both *in vitro* and *in vivo*, by using a mutant form of hIL-6 (Sant1) formulated in an adjuvant (aluminum hydroxide) compatible with human use. However, in the immunisation experiment described in the Example 5, the antigen was injected intraperitoneally, which is not an administration route commonly used for immunization in humans. The objective was to determine whether a similar immune response, with high titer antibodies against Sant1 and also against wt hIL-6 could be raised in NSE/hIL-6 transgenic mice using an administration route used for vaccination in humans.

Groups of 8-9 NSE/hIL-6 transgenic mice (plus ten non-transgenic siblings born in the same litters used as controls) were immunised intradermally (I.D.), an administration route in mouse which correspond to the sub-cutaneous (S.C.) administration route in humans, currently used for the administration of several vaccines as decribed in the state of the art. Again, 100 μg of antigen (either Sant1 or wild type hIL-6) formulated in aluminum hydroxide at 1 mg/ml in a total volume of 100 μl (100 μg of aluminum hydroxide) were used for each injection, using an immunization protocol identical to the one described in the Example 1. The second blood sample (taken after the second injection or first booster) and the third blood sample (taken after the third injection or second booster) were then tested for the presence of antibodies against the antigen used for the immunization by means of an "ELISA" method identical to the one already described in the Example 1.

To carry out objective measurements that are comparable for all animals the dilution of serum that gives a reading of 0.5 $O.D._{450}$ above the highest reading of the preimmune serum from the same animal has been conventionally termed "titer". The titer was measured as

illustrated in figures 1a, 1b, 2a and 2b for both normal and transgenic mice immunized with wild type hIL-6 and with Sant1. The results are reported in Table 8.

Again, as regards the mice imunized with the wt hIL-6 antigen, it can be seen again that (apart from the variation from one animal and another) on average the non-transgenic mice developed an antibody response against wt hIL-6 40-50 times stronger than that obtained in NSE/hIL-6 transgenic mice, this being proof of the fact that the transgenic mice have developed an immune tolerance to human IL-6 also when this antigen is injected formulated in aluminum hydroxide via intra-dermal administration route. On the contrary, as regards the mice immunized with Sant1 formulated in aluminum hydroxide, it can be seen that also in this case on average the non-transgenic mice developed an antibody response equivalent to that obtained in NSE/hIL-6 transgenic mice, as it was in the immunization experiments described in Examples 1 and 5, suggesting that the seven substitutions (which when introduced into hIL-6 generated Sant1) have rendered the mutant a completely foreign protein also when formulated in aluminum hydroxide and injected via intradermal administration route.

Table 8. Immunogenicity of Sant1 and of wt hIL-6 formulated in aluminum hydroxide and injected intradermally (I.D.) and NSE/hIL-6 transgenic and wild type control mice.

Mice group	mouse number	SERUM TITER 2nd sample	SERUM TITER 3rd sample
Non-transgenic	31	6220	4250
mice	45	888	1270
immunised	48	52	1150
with	50	1060	7640
wild type	55	4050	4560
hIL-6 in	57	4337	9260
aluminum	61	4065	9020
hydroxide	64	13690	20000
via	66	5460	7500
I.D.	65	680	700
Averages of the group		**4050**	**6535**
NSE/hIL-6	47	240	195
mice	52	51	25
immunised	62	30	120
with	81	39	160
wt hIL-6	86	97	200
in aluminum	91	77	100
hydroxide	96	306	210
via I.D.	2	25	100
Averages of the group		**108**	**139**
	25	890	4610
Non-transgenic	30	540	3670
mice	31	4370	13960

immunised	32	1770	10690
with	37	7860	13235
Sant1 in	39	8400	26680
aluminum	40	7360	49774
hydroxide	45	6510	4770
via I.D.	50	1000	16280
	53	470	3100
Averages of the group		**3920**	**14680**
	41	4640	5735
NSE/hIL-6	42	2145	7640
mice	47	1150	4490
immunised	48	1600	8700
with	49	2110	6740
Sant1 in	51	8980	20250
aluminum	52	11400	23380
hydroxide	56	2030	9880
via I.D.	65	4290	14080
Averages of the group		**4260**	**11210**

EXAMPLE 10

The antibodies developed in the serum of NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide are capable of recognising not only Sant1 but also wild-type hIL-6

This was to test whether or not the antibodies developed in NSE mice vaccinated with Sant1 formulated in aluminum hydroxide and injected *via* intradermal administration route were capable of cross-reacting with wild type hIL-6 (wt hIL-6). This was tested once again by "ELISA" using the same methodology described in the Example 2. The antibody titer was calculated as previously described and as illustrated in figures 1a, 1b, 2a and 2b, and the data obtained are reported in

Table 9.

Table 9 - Cross-reactivity against wt hIL-6 of sera of NSE/hIL-6 transgenic mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intradermally.

Mice group	mouse number	2nd sample serum titer wt hIL-6	2nd sample serum titer Sant1	3rd sample serum titer wt hIL-6	3rd sample serum titer Sant1
	41	960	4640	5800	5735
NSE/hIL-6	42	2720	2145	7820	7640
mice	47	665	1150	2190	4490
vaccinated	48	1010	1600	7075	8700
with	49	2660	2110	5060	6740
Sant1 in	51	6660	8980	17970	20250
aluminun	52	13380	11400	20740	23380
hydroxide	56	2360	2030	7240	9880
via I.D.	65	4290	4290	10700	14080
Averages of the group		**3856**	**4260**	**9400**	**11210**

Again, also when aluminum hydroxide is used as adjuvant for the immunization this time *via* intradermal administration route, the antibody titers against Sant1 and wild type hIL-6 are extremely similar. Once more, it should be noticed that when the NSE/hIL-6 transgenic mice are immunised with wild type hIL-6, the antibody response directed against wild type hIL-6 itself is much lower: for instance in the third blood sample the average titer against wt hIL-6 is 139 in the group of NSE/hIL-6 mice immunised with wt hIL-6 (see Example 9) as compared with an average titer against wt hIL-6 of 9400 (70 times higher) in the group of NSE/hIL-6 mice immunised with Sant1.

EXAMPLE 11

The antibodies developed in NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intradermally are also able to neutralise the biological activity of wild type hIL-6

The objective was to test whether or not these antibodies, developed in NSE/hIL-6 transgenic mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intradermally, are also capable of neutralising the biological activity of wild type hIL-6 itself.

Again, the biological activity of wild-type IL-6 under consideration was the ability to stimulate transcription by the C-reactive gene promoter in human Hep3B hepatoma cells. The effectiveness of transcriptional stimulation was measured according to the state of the art (Gregory, B., Savino, R. and Ciliberto, G., *J. Immunological Methods*, **170**, 47-56, 1994). As it was in the Example 7, human Hep3B hepatoma cells were stimulated with 4 ng/ml of wild-type hIL-6, and this extent of stimulation was taken as 100%, or with 4 ng/ml of wild type hIL-6 in the presence of serial dilutions of the serum obtained from the third blood sample from NSE/hIL-6 mice immunised intradermally with both Sant1 and wild type hIL-6; in the letter cases the extent of transcriptional stimulation was expressed as percent of the stimulation obtained in cells incubated with 4 ng/ml of wild type hIL-6 only. The results of the experiment are given in Figures 6a and 6b. It can be seen that the serum of all mice diluted 1:400 inhibits more than 80% of the biological activity of wild type hIL-6 at 4 ng/ml on human hepatoma cells. Again, it should be noticed that when NSE/hIL-6 transgenic mice are immunised with wild type hIL-6 the very low amount of anti hIL-6 antibodies obtained is not sufficient to inhibit wild type hIL-6 biological activity on human hepatoma cells (see Figure 6b).

Also in this case, investigations were carried out as to whether or not the occurrance of this cross-reactive immune response against wild type hIL-6 was capable of altering the levels of wild type hIL-6 measured in the serum of NSE/hIL-6 transgenic mice immunised with both Sant1 and wild type hIL-6. The hIL-6 levels were measured as described in the Example 3 both in the pre-immune sample and in the third sample of both groups of mice. The results are summarised in Table 10. As can be seen, vaccination using Sant1 formulated in aluminum hydroxide and injected intradermally, as well as causing the appearance of a strong antibody response that recognises both Sant1 itself and the wild type hIL-6, also causes an average decrease of about 350 times in the levels of hIL-6 that can be detected in the serum of the NSE/hIL-6 transgenic mice. On the contrary, immunisation using wild type hIL-6 itself formulated in the same adjuvant causes only a marginal decrease (2.4-fold as compared with 350-fold) in the levels of hIL-6 that can be detected in the serum.

Table 10 - Decrease in the levels of hIL-6 that can be detected in the serum of NSE/hIL-6 mice immunised with Sant1 and wt hIL-6 formulated in aluminum hydroxide and injected intradermally.

Mice group	mouse number	IL-6 levels in the serum (pg/ml) Pre-immune sample	3rd sample
NSE/hIL-6 mice immunised with **wt hIL-6** in aluminum hydroxide via I.D.	47	23790 pg/ml	5684 pg/ml
	52	25450 pg/ml	3110 pg/ml
	62	26190 pg/ml	4490 pg/ml
	81	24690 pg/ml	25930 pg/ml
	86	22890 pg/ml	8600 pg/ml
	91	16600 pg/ml	6884 pg/ml
	96	16760 pg/ml	5800 pg/ml
	2	22970 pg/ml	14670 g/ml

Averages of the group		**22418 pg/ml**	**9396 pg/ml**
NSE/hIL-6	41	21640 pg/ml	*9 pg/ml
mice	42	19040 pg/ml	*9 pg/ml
immunised	47	22940 pg/ml	173 pg/ml
with	48	23990 pg/ml	*9 pg/ml
Sant1 in	49	21640 pg/ml	79 pg/ml
aluminun	51	25680 pg/ml	21 pg/ml
hydroxide	52	24410 pg/ml	17 pg/ml
via I.D.	56	26540 pg/ml	204 pg/ml
	65	20410 pg/ml	78 pg/ml
Averages of the group		**22920 pg/ml**	**66 pg/ml**

The star (*) indicates the lower limit of sensitivity of the assay.

EXAMPLE 12

The antibodies developed in NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intradermally are able to neutralize the biological activity of wt hIL-6 also *in vivo*

The objective was to test whether or not these antibodies, developed in NSE/hIL-6 transgenic mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intradermally, are also capable of neutralising one of the biological activities of wild type hIL-6 *in vivo*, that is the increase of mouse SAA (mSAA) levels normally induced in the mice by injection of hIL-6, as described in the Example 4. The experiment was performed as described in the Example 4 on NSE/hIL-6 mice vaccinated with Sant1 formulated in aluminum hydroxide and injected intradermally. The values obtained have been compared with the control unimmunised mice of the experiments described in the Examples 4 (Table 3) and

8 (Table 7). The results are summarised in Table 11. As can be seen, apart from the variations from between one animal and another, on average again the 7-fold increase in SAA levels caused by hIL-6 injection in unimmumnised mice was absent in the mice vaccinated with Sant1 formulated in aluminum hydroxide injected intradermally.

Table 11 - Increase in the levels of mSAA detectable in the serum of Sant1-immunised and control NSE/hIL-6 transgenic mice after hIL-6 injection.

		mSAA levels in the serum		mSAA levels fold increase	
Mice group	mouse number	pre-inject. sample	post-inject. sample	single mouse	group average
	41	49 µg/ml	20 µg/ml	0.46	
	42	10 µg/ml	9 µg/ml	0.9	
	47	64 µg/ml	56 µg/ml	0.87	
Sant1	48	31 µg/ml	18 µg/ml	0.74	
vaccinated	49	8 µg/ml	18 µg/ml	2.2	**1.22**
	51	23 µg/ml	23 µg/ml	1.0	
	52	23 µg/ml	40 µg/ml	1.7	
	56	39 µg/ml	41 µg/ml	1.05	
	65	31 µg/ml	66 µg/ml	2.1	
	5	112 µg/ml	718 µg/ml	6.4	
	8	41 µg/ml	694 µg/ml	16.9	
	14	140 µg/ml	613 µg/ml	4.4	
	15	85 µg/ml	265 µg/ml	3.1	
	16	99 µg/ml	366 µg/ml	3.7	
	19	296 µg/ml	2997 µg/ml	10.1	
Control	20	41 µg/ml	146 µg/ml	3.6	**7.07**
unimmunised	21	47 µg/ml	479 µg/ml	10.13	
	21 bis	45 µg/ml	276 µg/ml	6.1	
	22	51 µg/ml	412 µg/ml	8.1	
	51	53 µg/ml	433 µg/ml	8.15	

55	41 μg/ml	303 μg/ml	7.4
56	75 μg/ml	291 μg/ml	3.9

Therefore, vaccination with Sant1 performed with an adjuvant and an administration route compatible with use in humans, as well as causing the appearance of a strong antibody response that recognises both Sant1 itself and the wthIL-6 and that is able to neutralize wt hIL-6 biological activity *in vitro* on human hepatoma cells, prevents *in vivo* the increase of mSAA levels induced by injection of hIL-6, in other words in neutralizes the biological activity of hIL-6 also *in vivo*.

WE CLAIM:

1. Use of a mutein of human interleukin-6, that is also a receptor antagonist of human interleukin-6, for the preparation of a pharmaceutical composition comprising said mutein as immunogen wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and wherein said mutein is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6, said pharmaceutical composition being suitable for the treatment of prevention of diseases caused by over-production of human interleukin-6 by vaccination.

2. Use of a mutein of human interleukin-6 according to claim 1, wherein said diseases are chronic auto-immune disorders, systemic lupus erythematosus, myeloma/plasmacytoma, post-menapausal osteoporosis and cancer cachexy.

3. Use according to claim 1 or 2, wherein said pharmaceutical composition additionally comprises a pharmaceutically acceptable carrier, diluent, excipient or vehicle.

4. Use, according to any one of claims 1 to 3, wherein said mutein is present in said pharmaceutical composition at a concentration of about 0.1 to 100 μg/ml.

5. Use, according to any one of claims 1 to 4, wherein said pharmaceutical composition is formulated with aluminium hydroxide at about 1mg/ml.

6. Use according to any one of claims 1 to 5, wherein said pharmaceutical composition is formulated for administration in three doses.

7. Use of a mutein of human interleukin-6 according to any one of claims 1 to 6, wherein said mutein is the mutein of human interleukin-6 (hIL-6), denominated Sant1, having the mutations Tyr 31 Asp, Gly 35 Phe, Ser 118 Arg, Val 121 Asp, Gln 175 Ile, Ser 176 Arg, Gln 183 Ala.

8. Use according to claim 7, wherein said mutein is Sant1 and said pharmaceutical composition further comprises an adjuvant that is aluminium hydroxide.

9. Use of a mutein of human interleukin-6 that is also a receptor antagonist of human interleukin-6, for treating or preventing diseases caused by overproduction of human interleukin-6, wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6.

10. Use of a mutein of human interleukin-6 according to claim 9, wherein said diseases are chronic auto-immune disorders, systemic lupus erythematosus, myeloma/plasmacytoma, post-menapausal osteoporosis and cancer cachexy.

11. Use of a mutein of human interleukin-6 according to claim 9 or 10, at a concentration of about 0.1 to 100 μg/ml.

12. Use of a mutein of human interleukin-6 according to any one of claims 9 to 11, wherein said mutein is the mutein of human interleukin-6, denominated Sant1, having the mutations Tyr 31 Asp, Gly 35 Phe, Ser 118 Arg, Val 121 Asp, Gln 175 Ile, Ser 176 Arg, Gln 183 Ala.

13. A vaccine for immunization against human interleukin-6, over-production of which causes a disease,

containing as an active immunogenic principal at least one mutein of human interleukin-6 that is also a receptor antagonist of human interleukin-6, wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and wherein said mutein is capable, upon vaccination, of eliciting production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6, in a pharmaceutically effective amount in a diluant, excipient or pharmaceutically acceptable vehicle.

14. The vaccine according to claim 13, wherein the disease is chronic auto-immune disorders, systemic lupus erythematosus, myeloma/plasmacytoma, post-menopausal osteoporosis and cancer cachexy.

15. The vaccine according to claim 13 or 14, containing as active immunogenic principal the mutein of human interleukin-6, denominated Sant1, having the mutations Tyr 31 Asp, Gly 35 Phe, Ser 118 Arg, Val 121 Asp, Gln 175 Ile, Ser 176 Arg, Gln 183 Ala.

16. The vaccine according to claim 15, containing as active immunogenic principle the mutein Sant1 formulated in aluminium hydroxide at 1mg/ml, formulated to be injected three times subcutaneously in the amount of 0.1-100 μg of mutein per injection.

17. A pharmaceutical composition comprising a mutein of human interleukin-6 that is also a receptor antagonist of human interleukin-6, and a pharmaceutically acceptable diluent, for use in treating or preventing a pathology caused by or related to over-production of interleukin-6, wherein said mutein comprises the mutations Tyr31Asp, Gly35Phe, Ser118Arg, Val121Asp, Gln175Ile, Ser176Arg, Gln183Ala and is capable, upon vaccination, of eliciting

production of antibodies that are able to neutralize the biological activity of wild-type human interleukin-6, and wherein said pathology is selected from the group consisting of chronic auto-immune disorders, systemic lupus erythematosus, myeloma/plasmacytoma, post-menopausal osteoporosis and cancer cachexy when said diseases are caused by an over-production of interleukin-6.

18. The pharmaceutical composition according to claim 17, wherein the pharmaceutical composition is capable of inducing an immune response effective to reduce the level of endogenous interleukin-6.

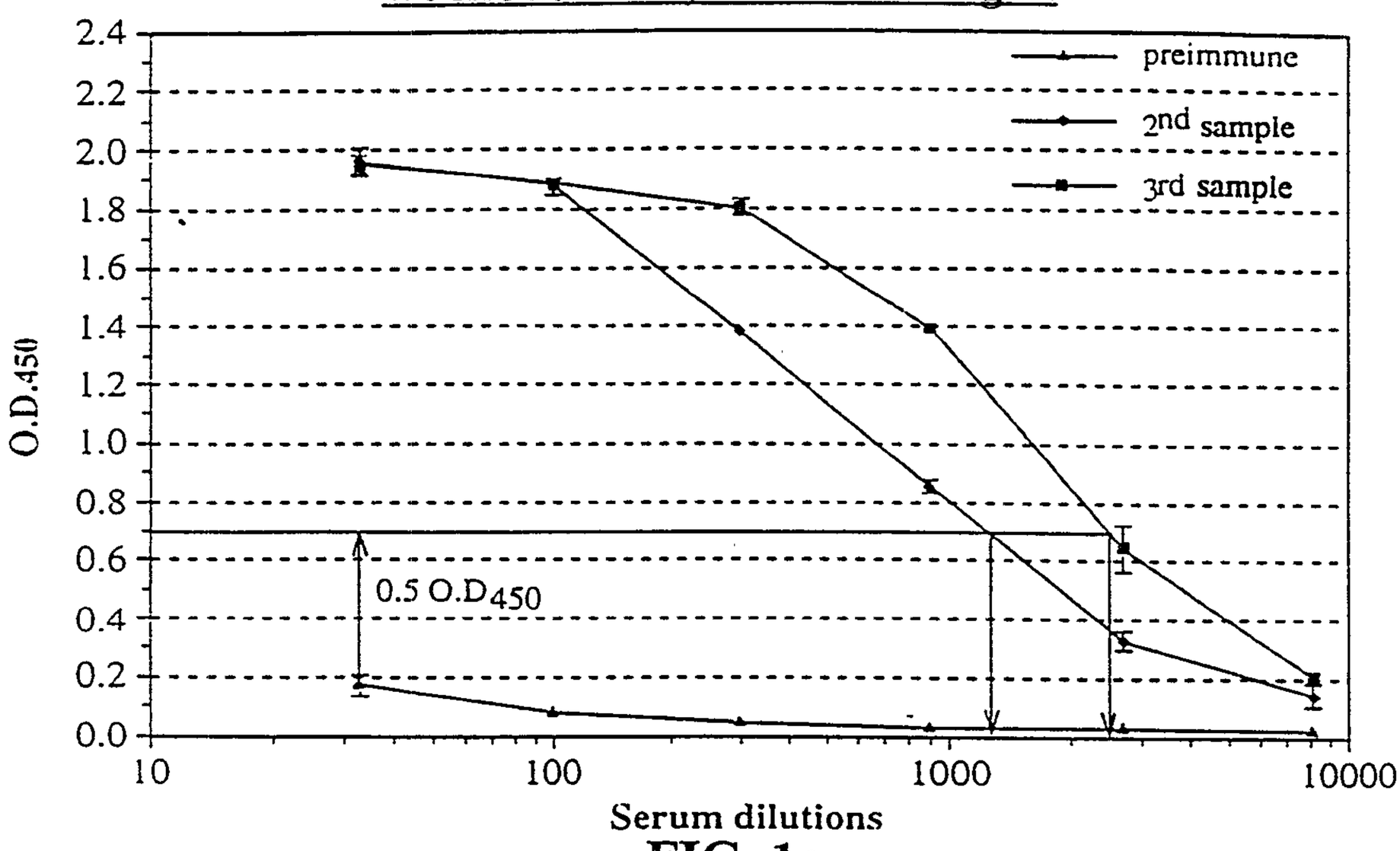
wt mouse #18; wt IL-6 antigen
preimmune
2nd sample
3rd sample
O.D.450
0.5 O.D450
Serum dilutions
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
10000

FIG. 1a

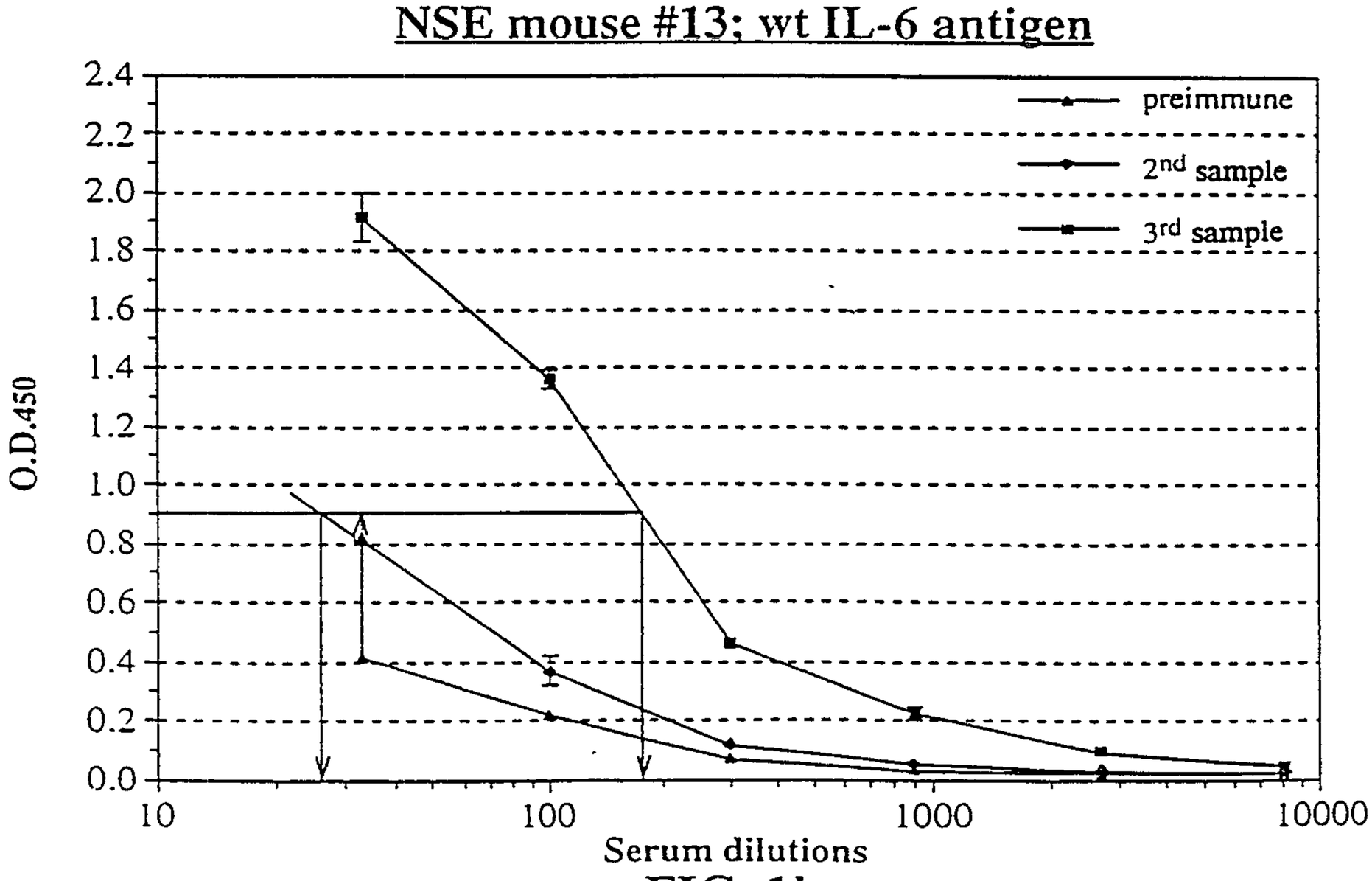
NSE mouse #13; wt IL-6 antigen
preimmune
2nd sample
3rd sample
O.D.450
Serum dilutions
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
10000

FIG. 1b

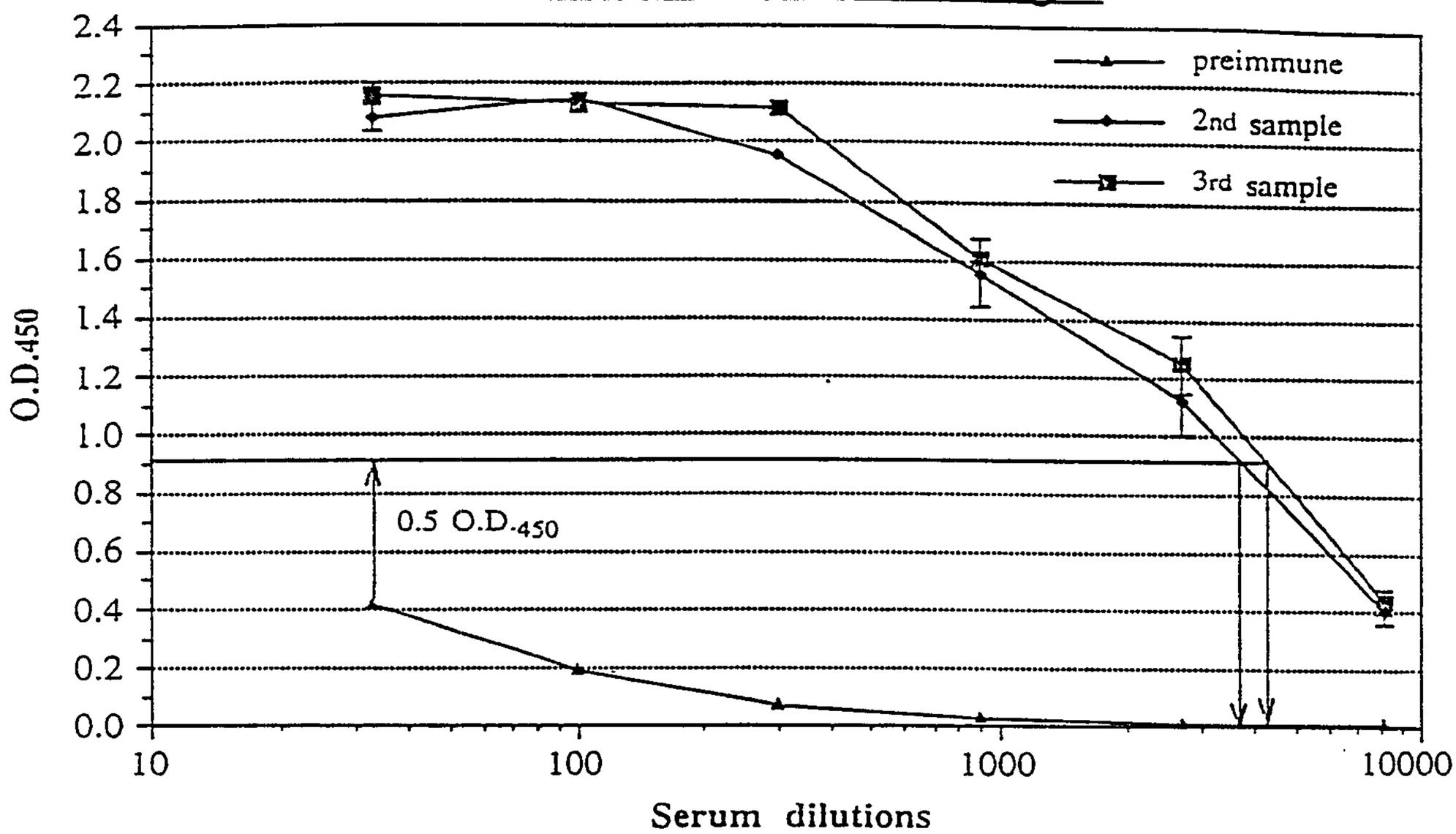
wt Mouse #10; Sant1 antigen
preimmune
2nd sample
3rd sample
0.5 O.D.-450
O.D.450
Serum dilutions
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
10000

FIG. 2a

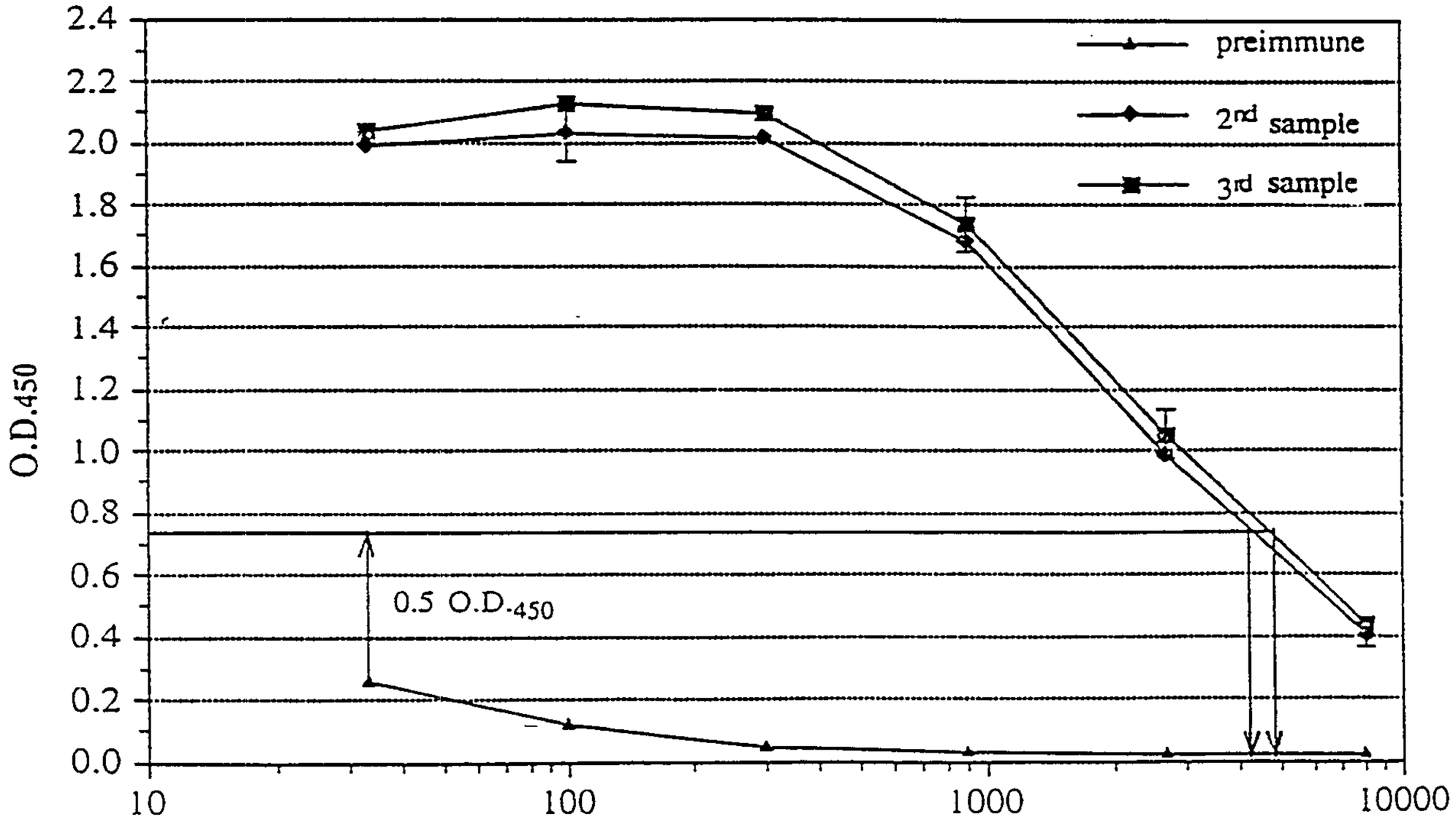
NSE mouse #1; Sant1 antigen
preimmune
2nd sample
3rd sample
0.5 O.D.-450
O.D.450
Serum dilutions
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
10000

FIG. 2b

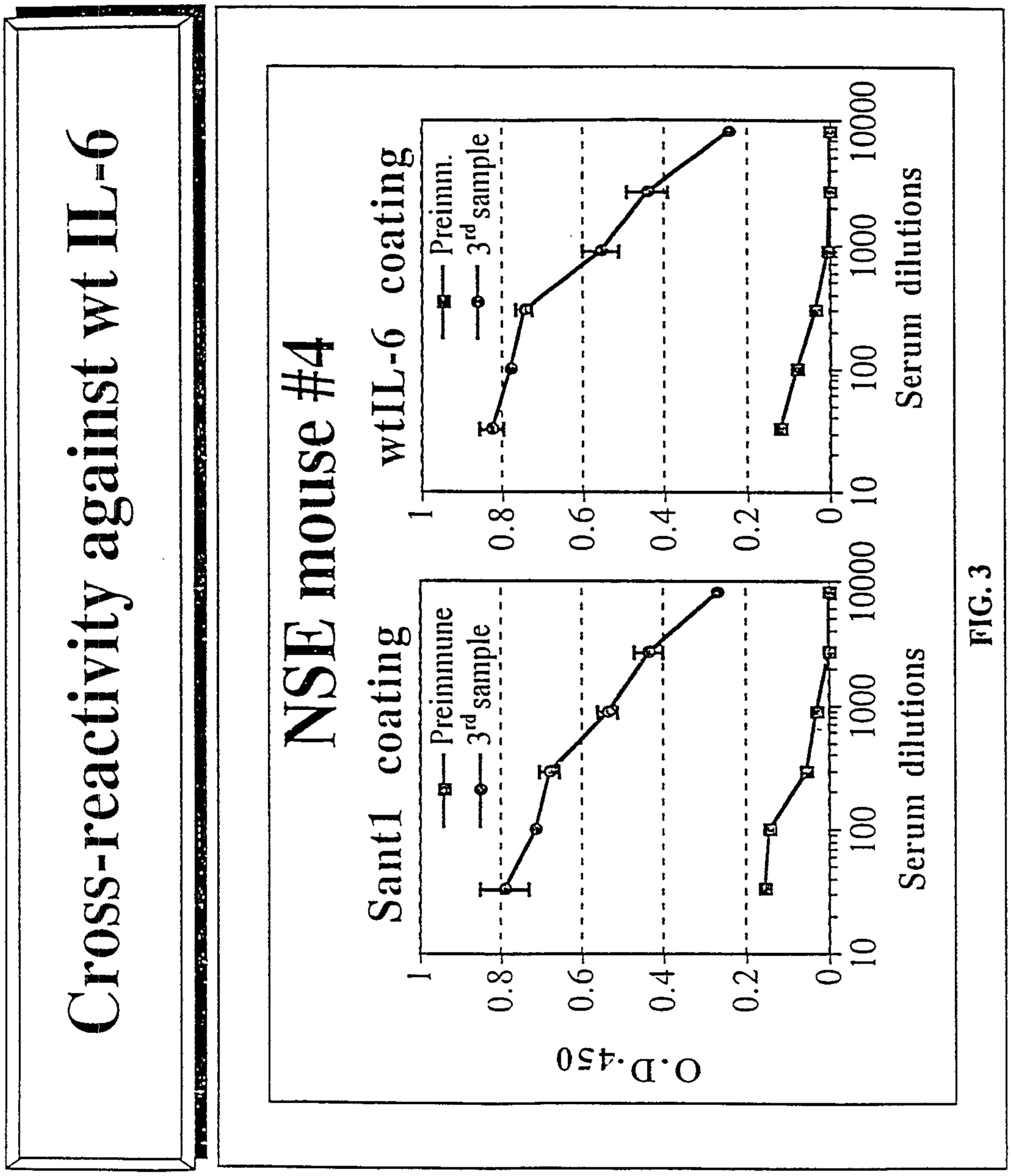
Cross-reactivity against wt IL-6
NSE mouse #4
wtIL-6 coating
Preimm.
3rd sample
Santl coating
Preimmune
3rd sample
O.D.450
Serum dilutions
1
0.8
0.6
0.4
0.2
0
10
100
1000
10000

FIG. 3

Cross-reactive Abs are neutralizing

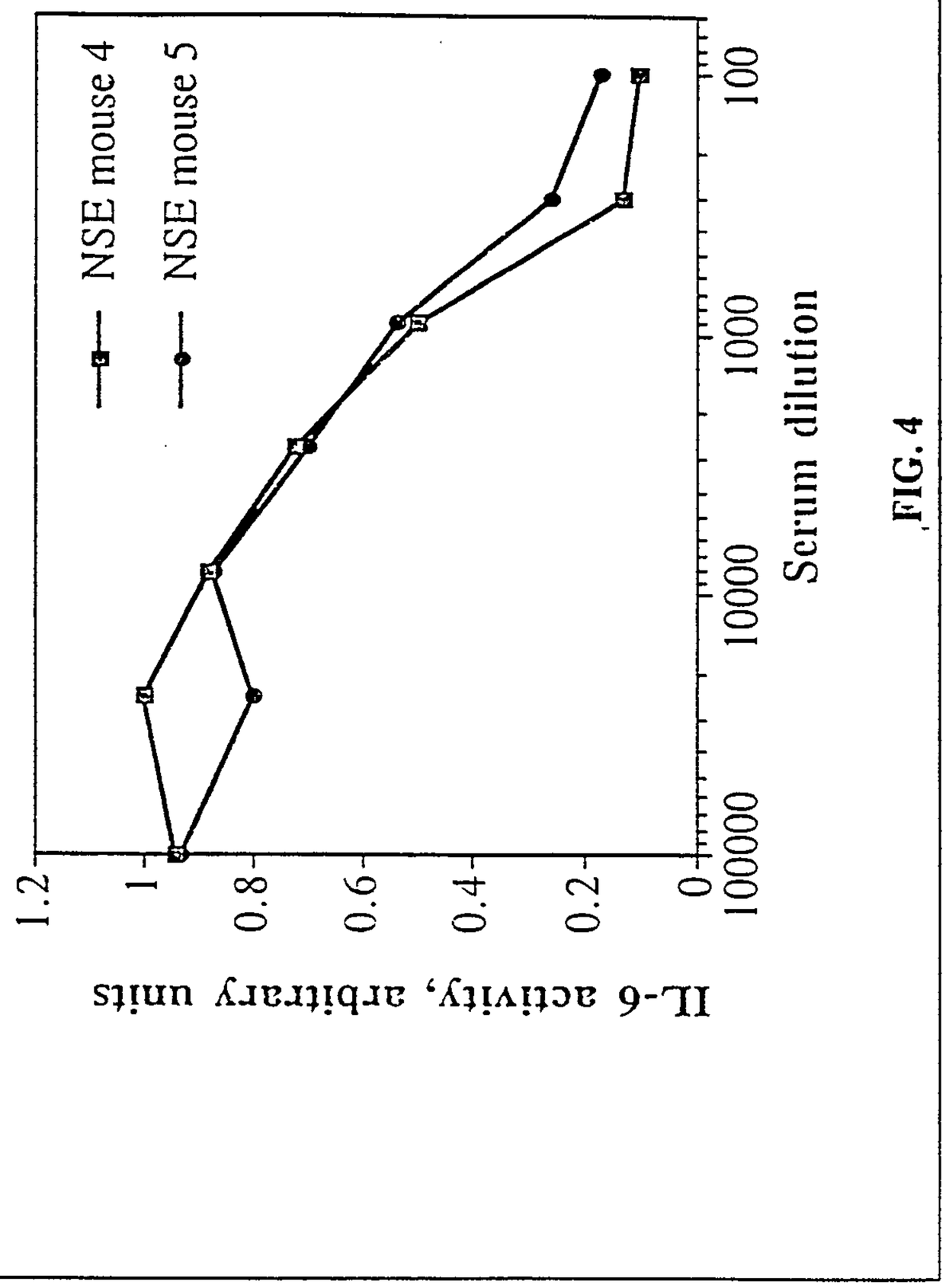

FIG. 4

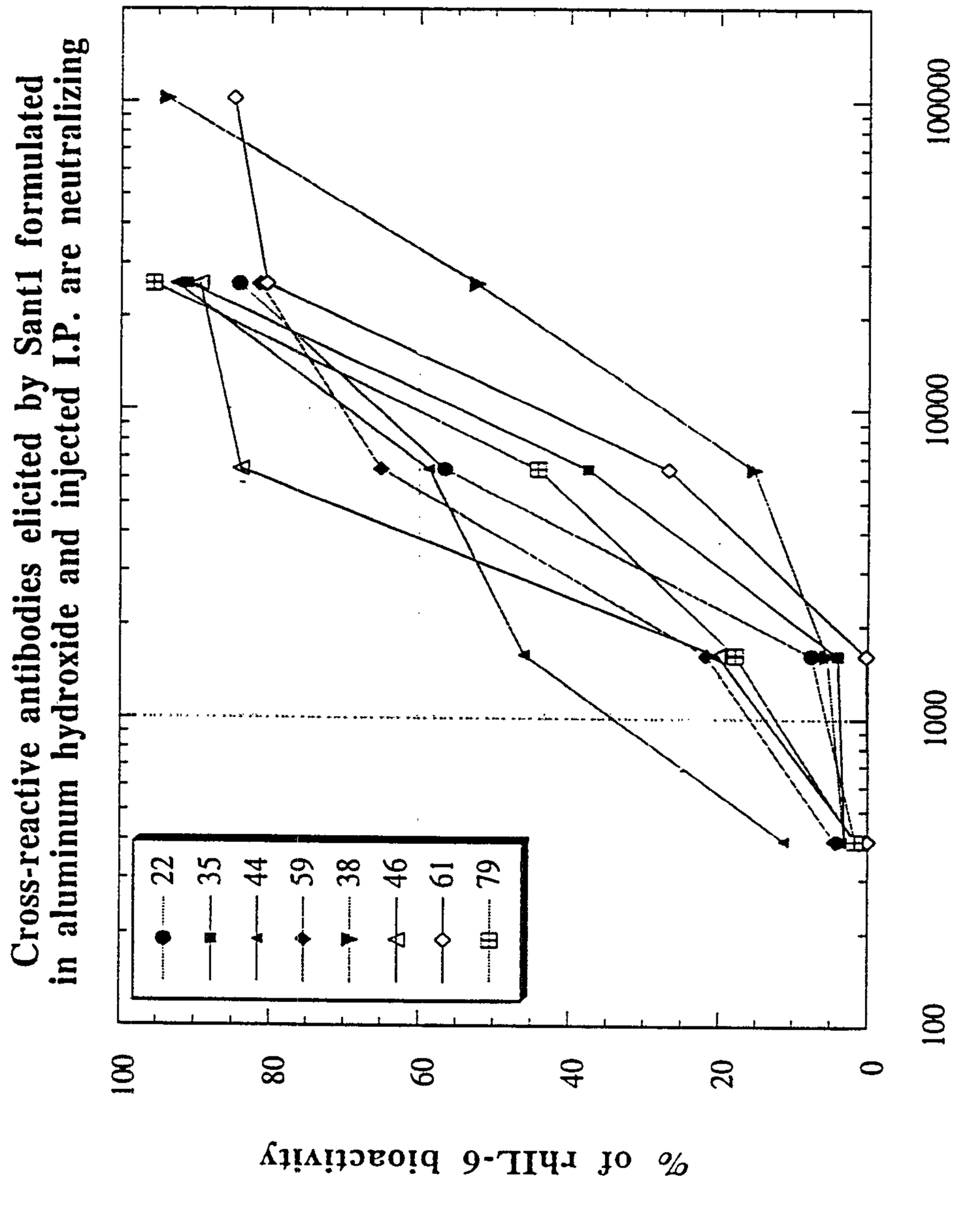
Cross-reactive antibodies elicited by Sant1 formulated in aluminum hydroxide and injected I.P. are neutralizing
% of rhIL-6 bioactivity
100
80
60
40
20
0
100
1000
10000
100000
serum dilutions
22
35
44
59
38
46
61
79

FIG. 5a

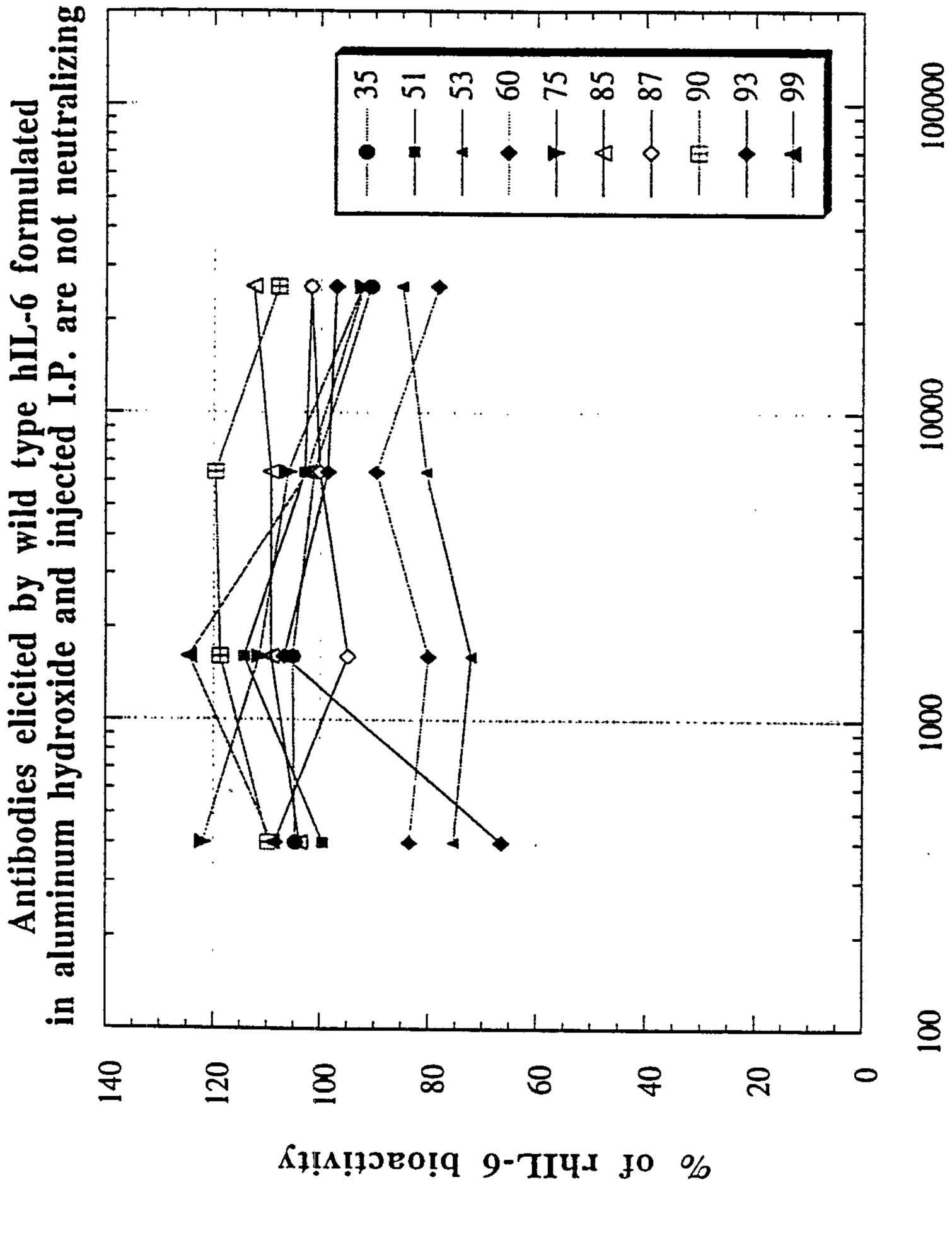
Antibodies elicited by wild type hIL-6 formulated
in aluminum hydroxide and injected I.P. are not neutralizing
35
51
53
60
75
85
87
90
93
99
% of rhIL-6 bioactivity
140
120
100
80
60
40
20
0
100
1000
10000
100000
serum dilutions

FIG. 5b

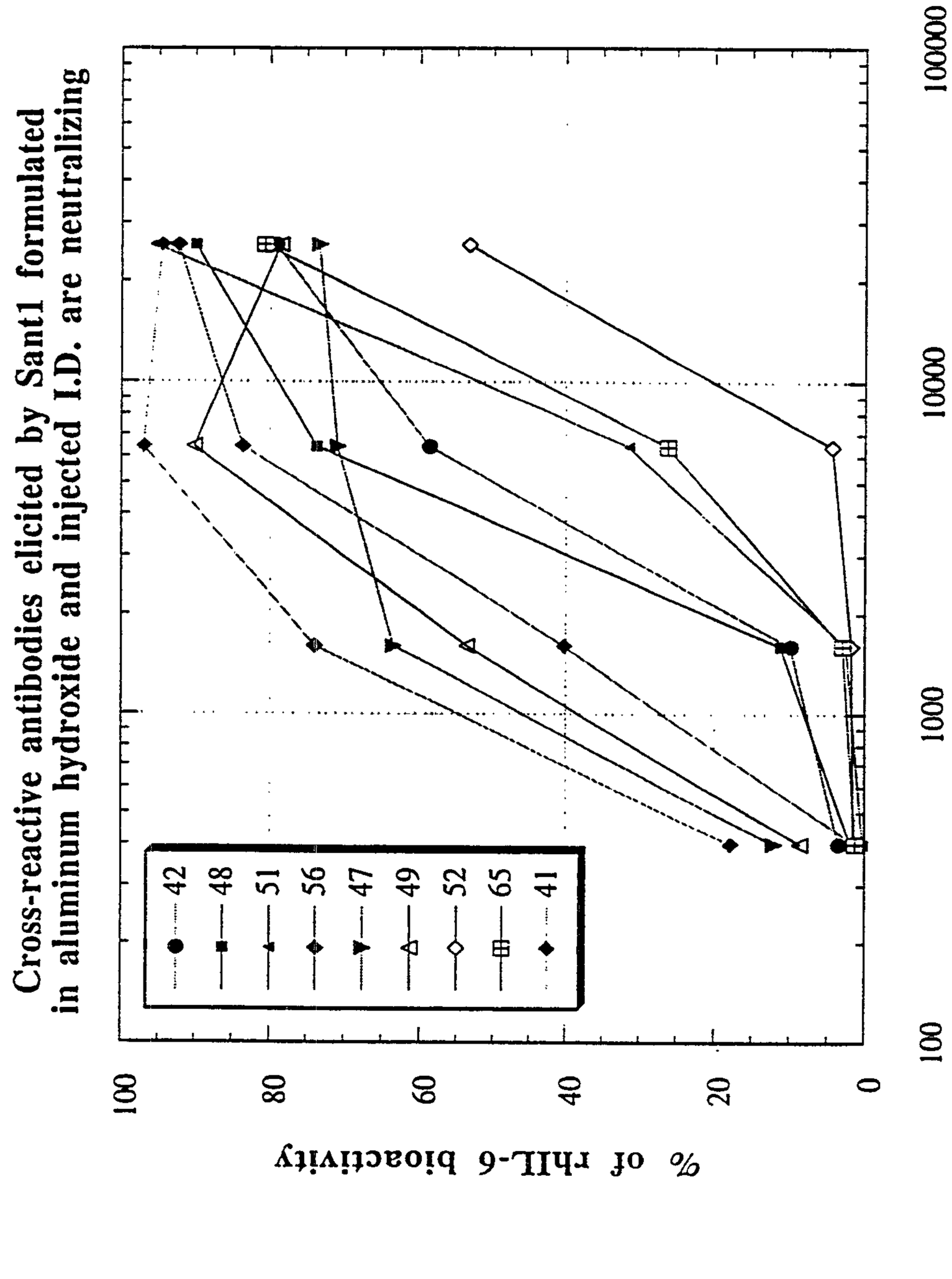
Cross-reactive antibodies elicited by Sant1 formulated in aluminum hydroxide and injected I.D. are neutralizing
% of rhIL-6 bioactivity
100
80
60
40
20
0
100000
10000
1000
100
serum dilutions
42
48
51
56
47
49
52
65
41

FIG. 6a

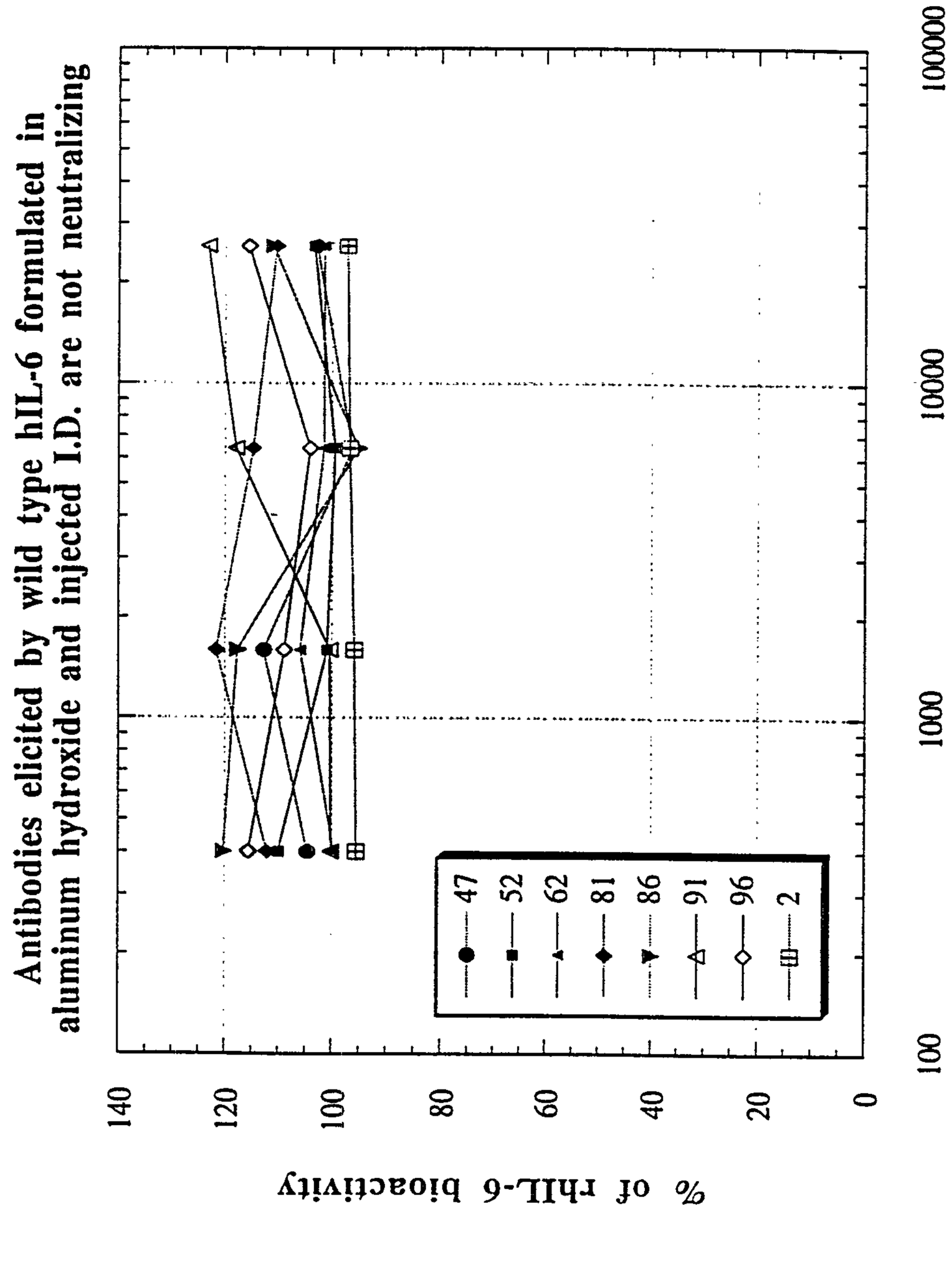
Antibodies elicited by wild type hIL-6 formulated in aluminum hydroxide and injected I.D. are not neutralizing
% of rhIL-6 bioactivity
140
120
100
80
60
40
20
0
100
1000
10000
100000
Serum dilutions
47
52
62
81
86
91
96
2

FIG. 6b