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(54) **CYTOKINES MODIFIEES POUR USAGE THERAPEUTIQUE**
(54) **MODIFIED CYTOKINES FOR THERAPEUTIC USE**

(57) L'invention concerne une nouvelle méthode d'utilisation thérapeutique des cytokines et de nouveaux produits pour la mise en oeuvre de cette méthode. La méthode est basée sur l'administration séquentielle d'un composant A (par exemple un antibiotique biotinylé) capable d'amener, vers une cible particulière atteinte d'une pathologie, un second composant B (par exemple de l'avidine), suivie par celle d'un composant capable de se lier au composant B comme par exemple la biotine (composant C), conjugué avec les cytokines. Le procédé permet d'augmenter la concentration locale en cytokines modifiées (cytokines C) par l'interaction avec le récepteur "artificiel" B et de provoquer une réponse biologique locale assurée par les récepteurs "naturels".

(57) The object of the present invention is a new method and new products for the therapeutic use of cytokines. The method is based on the sequential administration of a component A (e.g. a biotinylated antibody) able to deliver onto a particular pathologic target a second component B (e.g. avidin), followed by a component able to bind the component B (component C) (e.g. biotin), conjugated with the cytokines. The method permits to increase the local concentration of the modified cytokines (C-cytokines) through the interaction with the "artificial" receptor B and to cause a local biologic response mediated by "natural" receptors.

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(57) Abstract

The object of the present invention is a new method and new products for the therapeutic use of cytokines. The method is based on the sequential administration of a component A (e.g. a biotinylated antibody) able to deliver onto a particular pathologic target a second component B (e.g. avidin), followed by a component able to bind the component B (component C) (e.g. biotin), conjugated with the cytokines. The method permits to increase the local concentration of the modified cytokines (C-cytokines) through the interaction with the "artificial" receptor B and to cause a local biologic response mediated by "natural" receptors.

MODIFIED CYTOKINES FOR THERAPEUTIC USE

The present invention refers to modified cytokines for therapeutic use. The immune system produces cytokines and other humoural factors in response to different inflammatory stimuli, to traumas, to viral and bacteriological infections or to signals of cell degeneration, such as cancer. Although terms like "lymphokine", "monokines" and "cytokines" have been initially coined in order to distinguish products deriving from lymphocytes, monocytes and non-lymphoid cells, afterwards a sort of overlapping between these categories came out. Thus, the term "cytokines" is in current used as a synonymous of "lymphokines" and "monokines" and is hereinafter employed in this accepted meaning. A list of most of the cytokines known in the art, as well as a list of their biological activities, is reported in Aggarwal B.B. and Pocsik E.

It is well known that different cytokines exert antitumoral, antiviral and antibacteric activity. On the basis of such activities, observed both in vitro and in vivo in animal models, some cytokines have already been used therapeutically also in humans (De Vita et al., 1995, in Biologic Therapy of Cancer, Lippincott Company, Phyladelphia). For example, such cytokines as interleukine-2 (IL-2) and interferon α (IFNα) have shown positive antitumoral activity in patients with different types of tumors, such as kidney metastatic carcinoma, hairy cell leukemia, Kaposi sarcoma, melanoma, multiple mieloma, etc. Other cytokines like IFNβ, the Tumor Necrosis Factor (TNF) α, TNFβ, IL-1, 4, 6, 12, 15 and

the Colony Stimulating Factors (CFSs) have shown a certain antitumoral activity on some types of tumors and therefore are the object of further studies. Other cytokines have been used in the therapy of infective diseases (Aggarwal B.B. e Pocsik E.).

In general, the therapeutic use of cytokines is strongly limited by their systemic toxicity. Since this represents a crucial problem for their use in humans in therapeutically active amounts, many attempts have been made to develop new cytokines derivatives and new therapeutic strategies aimed at reducing the toxic effects of this class of biological effectors maintaining their therapeutic efficacy.

Tumor Necrosis Factor α (TNF) represents an emblematic case.

TNF is a cytokine, mainly secreted by macrophages, originally discovered for its capacity of inducing the hemorrhagic necrosis of some tumors (Carswell et al., 1975). Afterwards it has been demonstrated that TNF, besides exerting cytotoxic and cytostatic effects on different tumoral lines, can exert several other biological effects important for the regulation of the inflammatory and immune responses (Beutler and Cerami, 1989;Fiers, 1991).

The idea is now confirmed that TNF can exert healthy or toxic effects for the organism by which is produced, as a function of its concentration, of its production site and of the time of persistence in the site of action. For example, the chronic exposition to low amounts of TNF can provoke cachexy while the acute hyper-production of TNF can cause serious vascular

damages, shock and even death (Beutler and Cerami, 1989).

The potential antitumoral activity of TNF has been evaluated in various trials carried out on both animals and humans. Such trials have suggested that the antitumoral activity exerted in vivo by TNF mostly depends on its capacity of inducing damages to the tumor vascular system through direct effects on the endothelium and, in addition, through the activation of the inflammatory and immune responses (Sidhu and Bollon, 1993). On the contrary, less importance has been given to TNF-tumoral cells' direct cytotoxicity.

Although clinical trials (phase II) performed with TNF on different types of tumors have not shown a remarkably antitumoral activity, encouraging data have been obtained by using TNF associated with other drugs (IFN, IL-2, alkylating agents, Melphalan etc.). However it was generally noted that various types of TNF induced-toxic effects strongly limit the use of pharmacologically active amounts of the same (Spriggs and Yates, 1992). A fair success was instead obtained by using high amounts of TNF in regional therapy (e.g. through perfusion of limbs of melanoma affected patients) so as to reduce the systemic toxic effects (Lienard et al., 1992).

Different alternative therapeutic TNF-based strategies are now under evaluation, aimed at increasing therapeutic efficacy of TNF through an increase in the highest-tolerated amount and a reduction in the systemic toxic effects, obviously without jeopardize the antitumoral activity. It has been estimated that a

reduction of about one order of magnitude of the amount necessary for exerting an antitumoral effect could result well tolerated.

This was tried through:

a) the development of fusion proteins which can deliver TNF into the tumor and increase the local concentration. For example, the fusion proteins consisting of TNF and tumor specific-antibodies have been produced (Hoogenboom et al., 1991);

b) the development of TNF mutants which maintain the antitumoral activity and have a reduced systemic toxicity. Accordingly, mutants able of selectively recognizing only one receptor (p55 or p75) have been already prepared (Loetscher H et al., 1993).

c) the use of anti-TNF antibodies able to reduce some toxic effects of TNF without compromising its antitumoral activity. Such antibodies have been already described in literature (Rathien et al. 1992).

d) the use of TNF derivatives with a higher half-life (for example TNF conjugated with polyethylene glycol).

The pharmacologic potentials of such strategies are now under evaluation.

Although the data obtained encourage the use of TNF as an antitumoral agent, the problem of its systemic toxicity has not been solved yet.

Delivering systems for paramagnetic or radioisotopic cytotoxic agents employing the biotin-avidin interaction have been recently described (EP 251 494 and EP 496 074).

In particular, EP 251 494 describes a system for administering a diagnostic or therapeutic agent, which comprises: an antibody conjugated with avidin or streptavidin, an agent capable of complexing the conjugated antibody and a compound consisting of the diagnostic or therapeutic agent conjugated with biotin, which are administered sequentially and adequately delayed, so as to allow the localization of the therapeutic or diagnostic agent through the biotin-streptavidin interaction on the target cell recognized by the antibody.

The described therapeutic or diagnostic agents comprise metallic chelates, in particular chelates of radionuclides and low molecular weight antitumoral agents such as cis-platinum, doxorubicin, etc. In fact, it is expressly pointed out that the system is not suitable for compounds with molecular weights over 50,000 Daltons (preferably not over 10,000 daltons), and necessarily cannot be applied to cytokines, such as TNF (51,000).

EP 496 074 describes a method which provides the sequential administration of a biotinylated antibody, avidin or streptavidin and a biotinylated diagnostic or therapeutic agent. Also in this case, even though cytotoxic agents like ricin (a protein whose cytotoxic chain has a molecular weight of about 30,000 daltons) are mentioned, the application relative to radiolabelled compounds is mostly disclosed.

WO 95/15979 discloses a method for localizing highly toxic agents (I) on cellular targets, based on the administration of a first conjugate (C1) comprising

the specific target molecule conjugated with a ligand (L) or an anti-ligand (AL) followed by the administration of a second conjugate (C2) consisting of the toxic agent (I) bound to an anti-ligand (AL) or to the ligand (L). In this case, even though cytokines are cited among the toxic agents (I), including TNF, as well as the avidin/biotin system for L/AL, it can be deduced that when cytokines are used, the administration of the ligand (L) or (AL) is highly preferred for dissociating the cytokine thus allowing the "free" cytokine to exert the biological effects. This is likely due to the fact that the bound cytokine cannot react with its own receptors and exert efficaciously the desired biological effects. The necessary amount of L or AL is expected to be relatively high in order to compete for the binding on the cell surface, with possible consequent problems of toxicity. Moreover, when L/AL correspond to biotin/avidin, considering the high stability of such an interaction (the strongest non covalent known interaction, $Kd=10^{-15}$ M) such binding is practically indissociable (D. Savage et al., avidin-biotin chemistry: a handbook. Pierce Biotec Company (Rockford).

WO 95/15979 reports no specific experimental data which support the use of cytokines in the claimed method, but only generical citations that are not sufficient to provide the reproducible and practical teaching necessary for applying the method for localization to this class of substances that moreover do not act through a simple cytotoxic mechanism, but also through complex pro-inflammatory, immunostiulating, procoagulant and necrotizing mechanisms by which such

substances can exert antitumoral effects without exerting direct cytotoxic effects, as on the contrary it is required by using the agents of the above-mentioned applications EP-251 494 and EP-496 074.

Now, contrary to the generic indications reported in WO 95/15979, it has been surprisingly found that cytokines can be localized efficaciously in a biological active form only by means of a system in which the interaction between the conjugated cytokine and the target specific-conjugated component is not direct, as is the case of conjugation of a ligand/antiligand couple, but is rather mediated by a third component which can bind as a bridge between the target specific-component and the cytokine.

In accordance with the method of the present invention, the cytokine can also operate in a bound state and the administration of a member of the ligand/anti-ligand couple is not necessary in order to turn the cytokine in an active form. Such a result can be considered unexpected since it was unpredictable that the cytokines conjugated to a ligand could interact with the membrane receptors on the cell surfaces while interacting with the corresponding anti-ligand which in turn is involved in the interaction with the target specific component conjugated to a suitable ligand.

Thus the invention provides pharmaceutical compositions in the form of combined preparations for sequential therapeutic use, comprising:

a) an anti-pathologic target compound conjugated to a ligand of an at least ternary ligand/anti-ligand/ligand system;

b) an anti-ligand complementary to the ligand of compound a);

c) a cytokine conjugated to a ligand complementary to the anti-ligand b), with the proviso that the interaction ligand/anti-ligand/ligand is characterized by an affinity at least one order of magnitude higher than the affinity between the cytokine and its natural receptors.

Examples of compounds that can be conjugated to compound a) and to the cytokine, according to the invention, comprise haptens such as biotin and digoxigenin, while examples of "anti-ligand" compounds comprise anti-haptens antibodies (for example, anti-biotin antibodies and anti-digoxigenin antibodies) or, when biotin is used as a ligand, avidin and its analogues (e.g. streptavidin, neutravidin).

Preferably, both compound a) and the cytokine are conjugated to biotin, while avidin (or the analogue compound) is used as an anti-ligand.

The techniques used for the conjugation of the cytokines and of the antibodies are widely known and can be connected to chemical or to genetic engineering methodologies.

The anti-pathological target-compounds a) preferably are whole or fragmentary antibodies or monoclonal antibodies. Said antibodies have already been described and used widely, expecially in the case of antibodies directed against tumoral antigens.

Examples of cytokines or limphokines that can be used according to the invention comprise tumor necrosis factors, interferons, interleukins, colony stimulating

factors (CSF). It is also possible to use a biological response's modifier which can promote the local release of endogen cytokine, such as the lipopolysaccharide (LPS) or derivatives thereof, to obtain an increased local effect and lower systemic effects.

Moreover, as it is known that cytokines can sometimes exert additive or synergic effects, the strategy upon which the present invention is based can be performed in order to obtain synergic local effects and less effects at systemic level. The biologic therapy of cancer and the effects of the combination of different cytokines' are widely documented and well summarized by De Vita et al. 1995 (Biologic Therapy of Cancer, Lippincott Company, Phyladelphia).

Among cytokines the use of TNF is particularly preferred.

Preferably, the mutual interactions of the conjugates or their interactions with the artificial receptor will be characterized by affinity constants at least one order of magnitude higher than the affinity constants of the cytokines' membrane receptors and by kinetic dissociation constants at least one order of magnitude lower than that of interaction of cytokine with its natural receptors.

The anti-target compound or conjugated antibody can be locally administered or alternatively can be injected into the bloodstream, and can be reacted in vivo with the antigens or with the recognized cellular structures till the exceeding circulating compound or antibody is removed from the body, while a significant fraction remains bound to the pathological target. At this moment

the anti-ligand b) can be administered, followed by the conjugated cytokine in such a concentration that a bond with the antibody or anti-target compound can be formed thus allowing the mound or the increased persistence of the cytokines in the target cells.

This can be achieved if the dissociation time of the modified cytokine from the "artificial" receptor (represented by the anti-ligand) is at least one order of magnitude longer than the dissociation time from the "natural" receptors. As the examples relative to the applications of the invention demonstrate, it can be performed through a system consisting of a biotinylated antibody specific for a tumoral antigen, neutravidin, and biotin-TNF (bio-TNF). In this system the affinity between bio-TNF and the artificial receptor (avidin), equal to 10^{-15} M, and the affinity between bio-TNF and its natural receptors (TNF-R1 and TNF-R2), equal to 10^{-9} - 10^{-10} M, are such that the mound and the increased persistence of TNF are favoured in the site where the artificial receptors are present (tumor).

In the preferred embodiments of the invention, a biotinylated monoclonal antibody specific for a tumoral antigen is brought into contact with the tumor through its intra- or para-lesional, intra-cavity (e.g. bladder, peritoneal cavity), intra-artery (liver, central nervous system) administrations or at the systemic level, local or regional vascular perfusions (e.g. in the limb perfusional liquid, liver, breast), followed by analogue sequential administration of neutravidin or streptavidin and TNF-biotin.

Incubation times of some hours are preferably

inserted between each administration to allow the vascular system to eliminate the exceeding product thus obtaining a finer localization on the tumoral target.

Moreover, the use of TNF biotinylated at the amino terminal region (1-11 residues, VRSSSRTPSDK sequence) is preferred, so that the multivalent bond of TNF with its natural receptors is not hampered and its effects are not inactivated. Alternatively, amino acids bearing groups which can be easily biotinylated through known techniques (lysine, cysteine, tyrosine, hystidine, etc. - see Savage et al., Avidin-biotin chemistry: a handbook, Pierce Biotec Company), can be inserted in the same region through genetic engineering-techniques, or glycosylation signals, so that specific biotinylation of carbohydrate residues is obtained, for instance with biotin-hydrazide or derivatives thereof. The biotinylation of TNF at the amino-terminal portion can be easily obtained as well by means of genetic engineering-techniques through the construction of conjugates of TNF and fragments of proteins directly biotinylated by the expression system. An example of such proteins is represented by acetyl-CoA carboxylase from E. Coli, and by its C-terminal domain bearing the biotinylation site.

A biotinylation of the alpha-amino groups is preferred in order to keep the structure of biotin-TNF the closest to the structure of non-biotinylated TNF. This can be done by using protocols for biotinylation based on the reaction of biotin-6-aminocaproyl-N-hydroxysuccinimide ester at a pH comprised between 5.5 and 7.5. Alternatively, it was observed that

biotinylated TNF can be obtained such that the capability of interacting with avidin and with membrane receptors is maintained, by "mixing" the subunits from TNF for example biotinylated according to one of the above-described methods, and from non-biotinylated TNF. Preferably, the mixing reaction is carried out incubating mixtures of biotinylated and non-biotinylated TNF in a ratio of 1:3 for 24-72 hours, at 4°C. Such forms of TNF conjugated at the N-terminal are new and represent a further aspect of the invention.

Kits containing suitable therapeutic materials can be prepared in order to make the commerce and the routinary use of the compositions of the invention easier. Preferably, a kit according to the present invention comprises:

- a vial containing 0.5 to 10 mg of biotinylated antibody;
- a vial containing 5 to 100 mg of avidin or streptavidin or neutravidin;
- a vial containing 0.5 to 10 mg of biotinylated TNF (or other biotinylated cytokine).

The method of the present invention offers some advantages compared to known methods, for example the advantages deriving from the direct conjugation of cytokines with antibodies:

1) for example, it does not require the antibody affinity to be necessarily high, as it is possible that relatively high amounts of antibody and avidin are administered, followed by relatively lower amounts of biotin-TNF, and anyhow the tumor is efficiently labelled. On the other hand, this is

not possible with conventional conjugates antibody-TNF, for which the amount of antibody is invariably "linked" to TNF-amount.

2) The possibility of diffusion of the separated antibodies and TNF into the tumoral mass is presumably better than that obtainable with conjugates antibody-TNF, due to their lower molecular weight.

3) The tumor labelling with high affinity molecules like avidin and streptavidin (10^{-15} M) permits biotin-TNF amounts to be selected such that the interaction with the artificial receptor (avidin) is favoured thermodynamically and kinetically and the interaction with natural receptors at systemic level (10^{-9}-10^{-10} M) is hampered. In the case of antibody-TNF conjugates, it is extremely difficult to obtain antibodies able to bind the tumoral antigen with affinity in the order of 10^{-15} M.

The present invention is further described in the following examples.

Although the examples are particularly referred to the treatment of tumoral pathologies, it can be easily understood that the same strategy can be employed for the treatment of each other pathologies for which a local treatment with cytokines can be used.

EXAMPLE 1

This example shows an application of the invention based on the system:

Pathological target: A : B : C-cytokine

where:

Pathological target = murine lymphoma expressing the

murine antigen Thy 1.1 (RMA cells genetically engineered in order to express the allele Thy1.1 (RMA Thy 1.1 C1.2);

A= biotinylated anti-Thy 1.1 monoclonal, antibody (mAb bio-19E12).

B= neutravidin

C-cytokine= biotin-TNF conjugate

and where

(:) represents a non-covalent interaction, while

(-) represents a covalent bond.

Materials

- AH-BNHS (biotin 6-aminocaproyl-N-hydroxysuccinimide ester) (SPA, B002-61)
- SULFO-NHS-LC-Biotin (Pierce, 21335)
- Human TNFα (1x108 U/mg) (DRG080)
- Lysine (Sigma cod. L5501)
- Colture medium: sterile RPMI-1640 (Gibco 31870-025)
- Foetal Calf Serum (FCS) (PBI-Biological Industries, cod. 04-001-1A)
- Foetal Calf Serum (FCS) (Sigma, F2442)
- Geneticin (G418, Sigma cod. G9516) in 100 mM Hepes
- Hepes (Sigma, H0887)
- 200 mM Glutamine (Gibco cod. 04305030D)
- 10,000 IU/ml Penicillin, 10,000 mg/ml streptomycin, 25 µg/ml Amphotericin B (Gibco cod. 15240-021)
- NaCl (BDH cod. 10241)
- HCl 37% (BDH cod. 10125)
- 96-well flat-bottomed PVC plates for cell culture (Costar 3595)
- 96-well round-bottomed plates (PBI, 650180)
- 2-mercaptoethanol (Merck cod. 12006)

- Actinomycin D (Fluka 01815)
- Thiazolyl blue (MTT) (Merck 11714)
- PBS (NaCl 0.15 M, Na-phosphate 0.05 M, pH 7.3)
- Neutravidin (Pierce cod. 31000)
- Sodium azide (Baker, 9099)
- mAb 19E12

Example 1.1

Preparation of the biotinylated 19E12 mAb antibody (bio-19E12)

1000 µl of a solution of the 19E12 mAb, 1 mg/ml in sodium bicarbonate pH 8.5, 34 µl of a sulfur-NHS-LC-Biotin solution, 1 mg/ml (molar ratio mAb/biotin: 1/24) were pipetted into an Eppendorf tube. The mixture was incubated at room temperature (23/24°C) for 30 minutes. After incubation the mixture was dialyzed overnight against 2 litres of PBS at 4°C and kept at +4°C.

Example 1.2

Preparation of biotin-TNF (bio-TNF)

In this chapter some examples of the protocols used for the preparation of biotin-TNF conjugates are reported.

Example 1.2.1 (preparation of bio-TNF cod. #B)

60 µl of a solution of TNF (0.5 mg/ml) in bidistilled water, 6 µl of 1 M Na-carbonate pH 6.8, 6 µl of AH-BNHS, 3 mg/ml in DMSO (molar ratio TNF/Biotin: 1/66), were pipetted into an Eppendorf tube. The mixture was incubated at room temperature (23/24°C) for 3 hours and mixed with 7.5 µl of a 1 M lysine solution. After a further 1 hour incubation at room temperature 240 µl of the solution RPMI, 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 ug/ml streptomycin, 250 ng/ml amphotericin

B, were added to the mixture. The mixture was then dialyzed overnight against 2 litres of 0.9% NaCl at 4°C (3 changes) and kept at -20°C.

Example 1.2.2 (preparation of bio-TNF cod.#C, cod.#D, cod.#E, cod.#F, cod.#G)

The bio-TNF cod.#C product is prepared according to example 1.1.1 except for the use of a conjugation buffer at pH 7.8.

Comparative solutions of bio-TNF (cod.#D, cod.#E, cod.#F, cod.#G) are also prepared using different incubation buffers, various TNF/biotin molar ratios (see table 1).

Example 1.3

Determination of the biologic activity of biotinylated TNF on RMA Thy 1.1 C 1.2 cells.

This experiment is aimed at determining the specific biologic activity of different bio-TNF preparations obtained in different conjugation's conditions (see example 1.1), through a cytotoxicity test on RMA Thy 1.1 Cl.2 cells.

Procedure:

To each well of a 96-well flat-bottomed plate (Costar 3595) are added:

a) 60000 RMA Thy 1.1 Cl.2 cells (Mycoplasma free) in 50 µl of RPMI, 5% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycine, 250 ng/ml amphotericin B, 50 nM 2-mercaptoethanol, 500 µg/ml G418 (Complete-RPMI);

b) 50 µl of the standard TNF solution or of the solution of the sample in complete-RPMI at the desired concentration;

c) 10 µl of 330 ng/ml Actinomicyn D in Complete-RPMI.

Then the plate was incubated for 24 hours at 37°C, 5% CO2. Furthermore, 10 µl of a thiazolyl blue solution (MTT), 5 mg/ml in PBS, were added to each well. After further 4 hour incubation at 37°C, 5% CO2, 100 µl of lysis solution (33% (v/v) N,N-dimetylformamide), 20% (p/v) sodium dodecylsulfate, in water, brought to pH 4.7 with glacial acetic acid) were added to each well. The solutions were mixed in the wells with a multi-channel pipette and incubated for 24 h at 37°C. The absorbance of each well was then read by a multi-channel microplate reader at 570 and 650 nm (reference).

The cytotoxic activity was calculated by interpolation of the absorbances on a calibration curve obtained with non-biotinylated TNF.

Results

Table 1

Bio-TNF biologic activity, measured through cytotoxicity test on RMA cells.

		Biotinylation reaction		
	cod. (#)	TNF/biotin (molar ratio)	biologic activ. (pH)	activ.[a] (U/mg)
TNF	A	0 (non biotinyl.)	-	1.0×10^8
bio-TNF	B	1/66	6.8	5×10^7
bio-TNF	C	1/66	7.8	2.5×10^7
bio-TNF	D	1/66	8.8	3.1×10^7
bio-TNF	E	1/138	8.8	3.9×10^6
bio-TNF	F	1/275	8.8	3.0×10^5
bio-TNF	G	1/550	8.8	1.0×10^5

a) Measured using a calibration curve obtained with non-biotinylated TNF (cod.#A)

Example 1.4

Comparison of the binding and of the association and dissociation kinetics of bio-TNF and TNF from cells pre-treated with antibodies and neutravidin

The experiment is aimed at demonstrating that the constitution of artificial receptors on tumoral cells through the biotinylated antibodies and neutravidin pre-targeting system remarkably increases the total amount of bio-TNF which can bind to the cells compared to the maximum amount which can bind to natural receptors.

Moreover, this experiment is aimed at evaluating the association and dissociation times of bio-TNF with

natural and artificial receptors (neutravidin) from cells pre-treated with antibodies and neutravidin.

Procedure

The experiment was carried out treating MRA thy 1.1 Cl.2 cells as described in examples 1.5 and 1.6. Total bound TNF was detected using an indirect method based on the use of anti-TNF rabbit polyclonal antibodies and rabbit fluoresceinated goat anti-igG antibodies and FACS analysis.

Association kinetic

50,000 cells in 50 µl PBS/FCS 2% were seeded in the wells of a round bottomed plate, mixed with 1 µl of bio-19E12 mAb, 0.5 mg/ml in PBS/FCS 2% (antibody's final concentration 10 µg/ml), and incubated for 10 min on ice.

The cells were washed two times by adding 200 µl/well of PBS/FCS 2% and spinning for 2 min at 1300 rpm. The cells were resuspended by vortexing and mixed with 50 µl/well of 2% PBS/FCS and with 1 µl of 2.5 mg/ml neutravidin in PBS/FCS 2% (neutravidin's final concentration 50 mg/ml). After 10 min incubation on ice, cells were washed two times again with 2% PBS/FCS, as above.

50 µl of PBS/FCS 2%, 1 µl of TNF or bio-TNF at a concentration of 22.2 µg/ml (final concentration of TNF and bio-TNF 450 ng/ml) were then added to each well, and samples were incubated for 1 h on ice.

After a further washing with 2% PBS/FCS, 50 µl of rabbit anti-TNF polyclonal serum (Genzyme cod#IP300) 1:1000 in PBS/FCS 2% were added to each well, and incubated for 10 min on ice.

Cells were washed two times by addition of 2% PBS/FCS (200 µl/well) and centrifugation. Afterwards 50 µl of goat anti-rabbit immunoglobulin anti-serum conjugated with fluorescein (goat anti-rabbit-FITC) in PBS/FCS 2% were added to each well and incubated for 10 min on ice.

After the final washing with 2% PBS/FCS samples were resuspended in 200 µl of 2% PBS/FCS and analysed with FACS.

Dissociation kinetic

100,000 cells in 50 µl 2% PBS/FCS were seeded in 8 wells of a round bottomed plate, mixed with 1 µl of bio-19E12 mAb, 0.5 mg/ml in 2% PBS/FCS (antibody's final concentration 10 mg/ml), and incubated for 10 min on ice.

Cells were washed two times by adding 200 µl/well of 2% PBS/FCS and centrifuged for 2 min at 1300 rpm. Cells were resuspended by vortexing and mixed with 50 µl/well of 2% PBS/FCS and with 1 µl neutravidin 2.5 mg/ml in 2% PBS/FCS (final neutravidin concentration 50 µg/ml). After 10 min incubation on ice, cells were washed two times again with 2% PBS/FCS, as above.

50 µl of 2% PBS/FCS, 1 µl of TNF or bio-TNF at a concentration of 22.2 µg/ml (final concentration of TNF and bio-TNF 450 ng/ml), were then added to each well and the samples were incubated for 1 h on ice.

The 8 samples were washed with 200 µl of 2% PBS/FCS by centrifugation (twice). Cells were resuspended in 50 µl of RPMI, 5% FCS, 2 mM glutammine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and at different moments, fixed with

0.25% paraformaldehyde for 1 h at 4°C.

After a further washing with 2% PBS/FCS, 50 µl of rabbit anti-TNF polyclonal serum (Genzyme cod#IP300) 1:1000 in PBS/FCS 2% were added to each well, and incubated for 10 min on ice.

Cells were washed two times again through the addition of 2% PBS/FCS (200 µl/well) and centrifugation. Afterwards 50 µl of goat anti-rabbit immunoglobulin anti-serum conjugated with fluorescein (goat anti-rabbit-FITC) 1/12000 in 2% PBS/FCS and incubated for 10 min in ice.

After the final washing with 2% PBS/FCS the samples were resuspended in 200 µl of 2% PBS/FCS and analysed with FACS.

Results

The neutravidin pre-targeting on the cells provokes an at least 10-20 times higher increase in the bio-TNF binding to the cells, compared to the binding obtainable with the sole natural receptors (determined without neutravidin or using non-biotinylated TNF). The best increase was observed with bio-TNF cod.#B (biotinylated at pH 6.8) (data not shown).

Moreover, as it can be noted in figure 1 (lower panel), the time of persistence of bio-TNF on RMA-cells pre-treated with antibodies and avidin is about 30 times higher ($t_{1/2}$=7 h) then the persistence of the TNF on the same cells ($t_{1/2}$=0.22 h).

It can be concluded that the binding demonstrate that the exploitation of the antibody's and neutravidin's pre-targeting strategy in accordance with the invention, allows the maximum amount of bio-TNF

bound to tumoral cells to be increased 10-20 times and such as amount to persist on the cell membrane about 30 times longer.

Example 1.5

Comparison of TNF and bio-TNF citotoxicities on tumoral cells pre-treated with biotinylated antibodies and neutravidin, in vitro, in the presence of actinomycin D

The example is aimed at demonstrating the ability of a biotinilated antibody/avidin system to deliver biotin-TNF on the cell surface in the bilogical active form and to increase the in vitro citotoxicity of the same, compared to that obtainable simply using the natural receptors.

For this aim RMA Thy 1.1 Cl.2 cells, the human monoclonal anti-Thy 1 antibody, biotinylated (mAb bio-19E12), neutravidin, and bio-TNF cod#B have been used.

The experiment was conducted by sequentially incubating and washing the cells with a) mAb bio-19E12, b) neutravidin, c) bio-TNF.

As it is known that the TNF binding to natural receptors induces the synthesis of protective factors, such as Mn superoxidedismuthase, with the aim of inhibiting the production of such factors and of increasing the cytotoxic activity of TNF, the cytotoxicity test was carried out in the presence of a transcription inhibitor (actinomycin D).

Procedure

1,000,000 cells in 50 µl of 2% PBS/FCS and 1 µl of a solution of 0.5 mg/ml mAb bio-19E12 were added to each well of a 96-well round-bottomed plate (Costar 3595). The suspension was incubated for 10 min on ice. Cells

were washed two times by adding 200 µl/well of 2% PBS/FCS and spinning for 2 min at 1,300 rpm. Cells were resuspended by vortexing and mixed with 50 µl/well of 2% PBS/FCS and with 1 µl of 0.5 mg/ml neutravidin in 2% PBS/FCS. After 10 min incubation on ice, cells were washed two times again with 2% PBS/FCS, as above.

50 µl of 2% PBS/FCS, 1 µl TNF or bio-TNF at the desired concentration were then added to each well and incubated for 15 min on ice. After further washing with 2% PBS/FCS, cells of each well were resuspended in 1.5 ml of RPMI, 5% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, 50 nM 2-mecaptoethanol, 500 µg/ml G418 (complete-RPMI).

Cells were then seeded on 96-well flat-bottomed plates (60,000 cells/100 µl/well), mixed with 10 µl/well of a solution of 330 ng/ml actinomycin D in complete-RPMI, and incubated for 24 h at 37°C, 5% CO2.

The cell viability was determinated as described in the example 1.3.

Results

The results of the experiment conducted in the presence of actinomycin D are reported in fig. 2. It can be seen that, while in the absence of neutravidin the amount of TNF or bio-TNF necessary for killing 50% of the cells (LD50) (which can be detected from the 50% lowering of the absorbance) is higher than 100,000 U/ml, the LD50 of bio-TNF in the presence of neutravidin is about 2,000-3,000 U/ml. Furthermore, while the cytotoxicity of TNF is not influenced by the presence or by the absence of neutravidin, the cytotoxicity of bio-

TNF as it would be expected is strongly dependent on the presence of neutravidin. This shows that: a) TNF and bio-TNF are weakly cytotoxic for RMA cells in the absence of neutravidin, b) the presence of "artificial" neutravidin receptors on RMA cell membranes strongly increases the bio-TNF cytotoxicity, c) bio-TNF bound to the membrane through an antibody-biotin-avidin "bridge" can interact with its own natural receptors and trigger cytotoxic effects.

In an experiment carried out in the absence of actinomycin D, both TNF and bio-TNF have shown no cytotoxic activity at all (data not shown).

Example 1.6

Comparison of the in vivo tumorigenicity (mouse) of RMA Thy 1.1 cl2 cells pre-treated with biotinylated antibodies, neutravidin and with TNF or bio-TNF, in the absence of Actinomycin D

This experiment is aimed at demonstrating the reduced in vivo tumorigenicity of cells pre-treated with bio-TNF bound to the cellular surface through the antibody-biotin-neutravidin system (artificial receptor), compared to cells pre-treated with TNF or with bio-TNF exclusively bound to the natural receptors.

The adopted model is based on the subcutaneous administration of RMA Thy 1.1 Cl.2 cells pre-treated in vitro. The experiment was carried out using 4 groups of female C57 BL6 mice (5 mice for each group) and measuring the diameter of the tumoral mass at different days. Mice were treated with pre-treated cells as shown in table 2.

It is important to remember that the amounts of TNF

and bio-TNF used in this study (respectively 50,000 U/ml and 10,000 U/ml) are such as to determine partial cytotoxic effects only in the presence of actinomycin D, as demonstrated in the example 1.5 (fig. 2). In the present experiment cells were treated in the total absence of actinomycin D, i.e. in conditions according to wich both TNF and bio-TNF are not cytotoxic.

Table 2

Experimental scheme for the treatment of RMA Thy1.1 Cl.2 cells injected into the mice.

Group (#)	mice (N.)	mAb bio-19E12 (10µg/ml)	Neutrav. (10µg/ml)	TNF (50000U/ml)	bio-TNF (1000U/ml)
1	5	-	-	-	-
2	5	+	-	-	-
3	5	+	+	+	-
4	5	+	+	-	+

Procedure:

180,000 cells in 50 µl 2% PBS/FCS were seeded in four wells of a round bottomed plate, mixed with 1 µl of bio-19E12 mAb, 0.5 mg/ml in 2% PBS/FCS, and incubated for 10 min on ice.

Cells were washed two times by adding 200 µl/well of 2% PBS/FCS and spinning 2 min at 1,300 rpm. Cells were resuspended by vortexing and mixed with 50 µl/well of 2% PBS/FCS and with 1 µl of neutravidin 0.5 mg/ml in 2% PBS/FCS. After 10 min incubation on ice, cells were washed two times again with 2% PBS/FCS, as above.

Then, 50 µl of 2% PBS/FCS, 1 µl of TNF or bio-TNF at a respective concentration of 50,000 and 10,000 U/ml,

were added to each well, and incubated for 15 min on ice.

After further washing with 2% PBS/FCS, cells from each well were resuspended in 200 µl PBS, transferred to Eppendorf tubes, and mixed with 1.2 ml PBS. By the aid of syringes for insulin, 30,000 cells/200 µl/mouse were injected subcutaneously at the inguinal level. The mean diameter (lateral and longitudinal) of the tumoral mass was determined for different times using a calibre.

Results:

The results are reported in table 3. It can be noted that the cell treatment with bio-19E12/neutravidin/bio-TNF mAb (group 4), determined a reduction of the RMA Thy1.1 Cl.2 cell tumorigenicity in mice, compared to mice treated the same way but without bio-TNF (group 2). Differently the pre-treatment with bio-19E12/neutravidin/TNF mAb (group 3) did not produce a significative reduction of the tumorigenicity compared to the control group (group 2). In a further experiment carried out with 50,000 U/ml bio-TNF, instead of 10,000, the volume of the tumoral mass after 17 days was lower than the detection limit (15 mm^3) (data not shown).

These results indicate that bio-TNF bound to the cell membrane through the antibody/biotin/neutravidin "bridge" can trigger such effects that the RMA Thy 1.1 Cl.2 cell in vivo tumorigenicity is reduced.

Furthermore it is interestingly observed that although the amount of bio-TNF used for the treatment of the RMA cells was such to determine the cellular lysis, in the presence of actinomycin D (see the example 1.5), i.e. in such conditions that TNF cannot exert direct

cytotoxic effects, nevertheless the treatment provoked a reduction 5 times higher of the volume of the tumoral mass, after 17-19 days from the injection. This suggests that the reduced tumorigenicity is due to the host's indirect effects on the tumoral cells (inflammatory, immune effects, etc.).

Table 3

Diameter and volume of the tumoral masses in four groups of mice treated according to the scheme reported in table 2 at different days from the injection

Group	Mice (N.)	Diameter[a] (mm) day 11	day 14	day 15	day 19	Volume (mm^3) day 19
Group 1	1	-	5	7	12	
	2	-	5	8	15	
	3	3	-	-	15	
	4	5	7	10	15	
	5	3	3	5	10	
	mean	3.6	5	7.5	10.3	1231
	±SD	±0.64	±1.63	±2.1	±2.3	±194
Group 2	1	6	9	11	15	
	2	6	9	9	15	
	3	6	8	9	15	
	4	6	8	11	14	
	5	6	8	10	15	
	mean	6	8.4	10	14.8	1696
	±SD	±0.0	±0.5	±1.0	±0.44	±50
Group 3	1	6	8	9.5	15	
	2	7	7	8	15	
	3	6	8	10	15	
	4	6	7	10	15	
	5	5	8	11	15	
	mean	6	7.6±0.54	9.7	15	1766
	±SD	±0.7	±0.5	±1.09	±0.0	±0.0

Table 3 (continue)

Diameter and volume of the tumoral masses in four groups of mice treated according to the scheme reported in table 2 at different days from the injection

Group	Mice (N.)	Diameter[a] (mm) day 11	day 14	day 15	day 19	Volume (mm^3) day 19
Group 4	1	3	3	4.5	7.5	
	2	3	3	5.5	9	
	3	3	5	8	9	
	4	-	-	ascite	ascite	
	5	-	4	4	9	
	mean	3	3.75	5.5	8.6	322
	±SD	±0.0	±0.95	±0.88	±0.75	± 30

a) mean of longitudinal and lateral diameters.

Example 2

This example shows an application of the invention based on the system:

Pathological target: A: B: C-cytokine release-inducer

where:

Pathological target= murine lymphoma expressing the human Thy1.1 antigen (RMA Thy 1.1 C1.2 cells);

A= biotinylated human anti-Thy 1 monoclonal antibody (bio-19E12 mAb).

B= neutravidin

C-cytokine release-inducer= biotin-lipopolysaccharide conjugate (LPS)

and where

(:) represents a non-covalent interaction, while

(-) represents a covalent bond.

Example 2.1

Preparation of LPS-biotin

Materials

Lipopolysaccharide from Salmonella minesota (LPS): Sigma cod. L2137 lot. 101H4029

Biotinamidocaproylhydrazide: Sigma B-3770

Tris base: BDH cod. 10235

Sodium-M-periodate: Sigma S-1878

Sodium-acetate: BOM cod. 10235

Sodium-azide: J.T. Baker cod.9099

Solutions

- "Stop solution": 0.1 M Tris-HCl pH 7.5 (in water)
- Labelling solution": 100 mM Na-acetate, 0.02% Na-azide, pH 5.5.

Method

100 µl LPS (0.8 mg/ml in labelling solution) were mixed with 50 µl Na-M-periodate (30 mM in labelling solution). The mixture was incubated at room temperature in the dark for 30 min. After incubation the mixture was dialyzed against distilled water (4 changes of 1 h/each).

50 µl biotin-hydrazide (0.5 mg/ml in labelling solution) were added to the sample and the mixture was incubated for 1 h at room temperature.

50 ml of "stop solution" were added to the sample. The mixture was dialyzed against distilled water (4 changes of 1 h/each) and kept at -20°C.

Example 2.2

Control of the preparation of LPS-biotin

Materials

Sodium chloride (NaCl), BDH cod. 10241.

($NaH_2PO_4*H_2O$): BDH, cod. 102454R.

Sodium hydroxide (NaOH): BDH, cod. 10252.

BSA, bovin albumin: Sigma A4503.

Tween 20 (polyoxyethylene(20)sorbitanmonolaurate: BDH lot. ZA1645815.

Streptavidin: SPA (Società prodotti antibiotici) SAS1-610.

STV-HRP: Sigma S 5512.

OPD tablet, 5 mg: Sigma P 6912.

H_2SO_4: BDH 10276-5G

H_2O_2: Carlo Erba A902011404.

Solutions

- PBS: 0.15 M NaCl, 0.05 M Na-phosphate pH 7.3.
- PBS-3% BSA
- PBS-0.5% BSA-0.05% Tween
- PBS-0.05% Tween
- OPD: 2 tablets in 15 ml distilled water and 20 ml H_2O_2.
- 10% H_2SO_4.

Method

The preparation of LPS-biotin was incubated in PVC microplate (Becton Dickinson cod. 3912) with 10 µg/ml streptavidin in PBS, 100 µl/well, 1 h at 37°C. The plate was washed three times with PBS, then it was blocked with PBS-3% BSA, 200 µl/well for 2h at room temperature.

Serial dilutions (1:2) of biotin-LPS in PBS-0.5% BSA-0.05% Tween were prepared. The plate was washed

three times again with PBS.

100 µl/well sample were added, incubating 1 h at 37°C.

The plate was washed three times with PBS. 100 µl streptavidin-HRP 1:2000 in 0.5% PBS/BSA / 0.05% Tween were added to each well. After 1 h incubation at 37°C, the plate was washed three times with 0.05% PBS-Tween. 100 µl of OPD solution were added to each well.

The reaction was blocked with 100 µl of 10% H_2SO_4.

Results

Measured optical density

Biotin-LPS (µg/ml)	Optical density (492 nm)
0.3	1.150
0.1	1.107
0.03	0.986
0.01	0.670

Example 2.3

Comparison of the in vivo tumorigenicity (in mouse) of RMA Thy 1.1 Cl.2 cells pre-treated with biotinylated antibodies, neutravidin and with LPS or LPS-biotin

This experiment is aimed at demonstrating the reduced in vivo tumorigenicity of cells pre-treated with bio LPS bound to the cellular surface through the antibody-biotin-neutravidin system (artificial receptor), compared to cells pre-treated with LPS.

The employed model is based on the s.c. administration of RMA Thy 1.1 Cl.2 cells pre-treated in vitro. The experiment was carried out using 3 groups of C57 BL6 female mice (5 mice each) and measuring the diameter of the tumoral mass at different days. Mice were treated with pre-treated cells as indicated in

table 4.

Table 4

Experimental scheme for the treatment of the RMA Thy 1.1 Cl.2 cells injected into mice.

Group (#)	mice (N.)	mAb bio-19E12 (10 µg/ml)	Neutrav. (10 µg/ml)	LPS (2 µg/ml)	bio-LPS (2 µg/ml)
1	5	+	+	-	-
2	5	+	+	+	-
3	5	+	+	-	+

Procedure

The experimental procedure is identical to that reported in the example 1.6 except for the use of LPS or biotin-LPS respectively in place of TNF and biotin-TNF.

Results

The results are reported in table 5. As it can be noted, the treatment of the cells with bio-19E12/neutravidin/bio-LPS mAb (group 3) determined the reduction of the tumorigenicity of the RMA Thy 1.1 Cl.2 cells in mice, compared to the mice treated the same way but without bio-LPS (group 1) or with LPS (group 2).

The results indicate that the bio-LPS bound to the cellular membrane through an antibody-biotin/neutravidin "bridge" can trigger effects such that the tumorigenicity of the RMA Thy 1.1 Cl.2 cells is reduced in vivo.

RMA Thy 1.1 non-treated fresh cells were injected into the other flank of the two mice survived after the thirtieth day to verify any immune responses triggered by the previous treatment. As it can be noted in table

5, the two mice survived also after the second injection. Since the injected cells were not treated to the first injection, these results prove that the first treatment induced an immunologic memory.

Table 5

Per cent survival among the three groups of mice treated according to the scheme reported in table 4 after some days from the injection

	Day after the first injections										
Group	**10**	**18**	**19**	**20**	**21**	**22**	**25**	**30[a)]**	**40**	**45**	**60**
1	100	60	0	0	0	0	0	0	0	0	0
2	100	100	40	20	20	0	0	0	0	0	0
3	100	100	100	80	80	60	60	40	40	40	40

a) Second injection: non-treated fresh RMA Thy 1.1 cells were administered to the two survived mice.

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CLAIMS

1. Pharmaceutical compositions in form of combined preparations for sequential therapeutic use comprising:

a) an anti-pathologic target compound conjugated with a ligand of an at least ternary ligand/anti-ligand/ligand system;

b) an anti-ligand complementary to the ligand of the compound a);

c) a TNF conjugated at the amino-terminus with a ligand complementary to the anti-ligand b), with the proviso that the ligand/anti-ligand/ligand interaction is characterized by an affinity at least one order of magnitude higher than the affinity between the cytokine and its natural receptors.

2. Compositions according to claim 1 wherein biotin is the ligand and avidin, streptavidin or neutravidin are the anti-ligand.

3. Compositions according to claims 1 or 2, wherein both the compound a) and the TNF are conjugated with biotin and avidin, streptavidin or neutravidin are the component b).

4. Compositions according to any of the previous claims, wherein an antibody is the anti-pathologic target.

5. Compositions according to claim 4, wherein the antibody is a monoclonal antibody.

6. Composition according to claims 4 or 5, wherein the antibody is an antibody directed against tumoral antigens.

7. Tumoral necrosis factor conjugated with a ligand at the amino terminal.

8. Tumoral necrosis factor according to claim 7, wherein the ligand is conjugated with the α-amino group of the residue 1.

9. Tumoral necrosis factor according to claims 7 or 8, wherein biotin is the ligand.

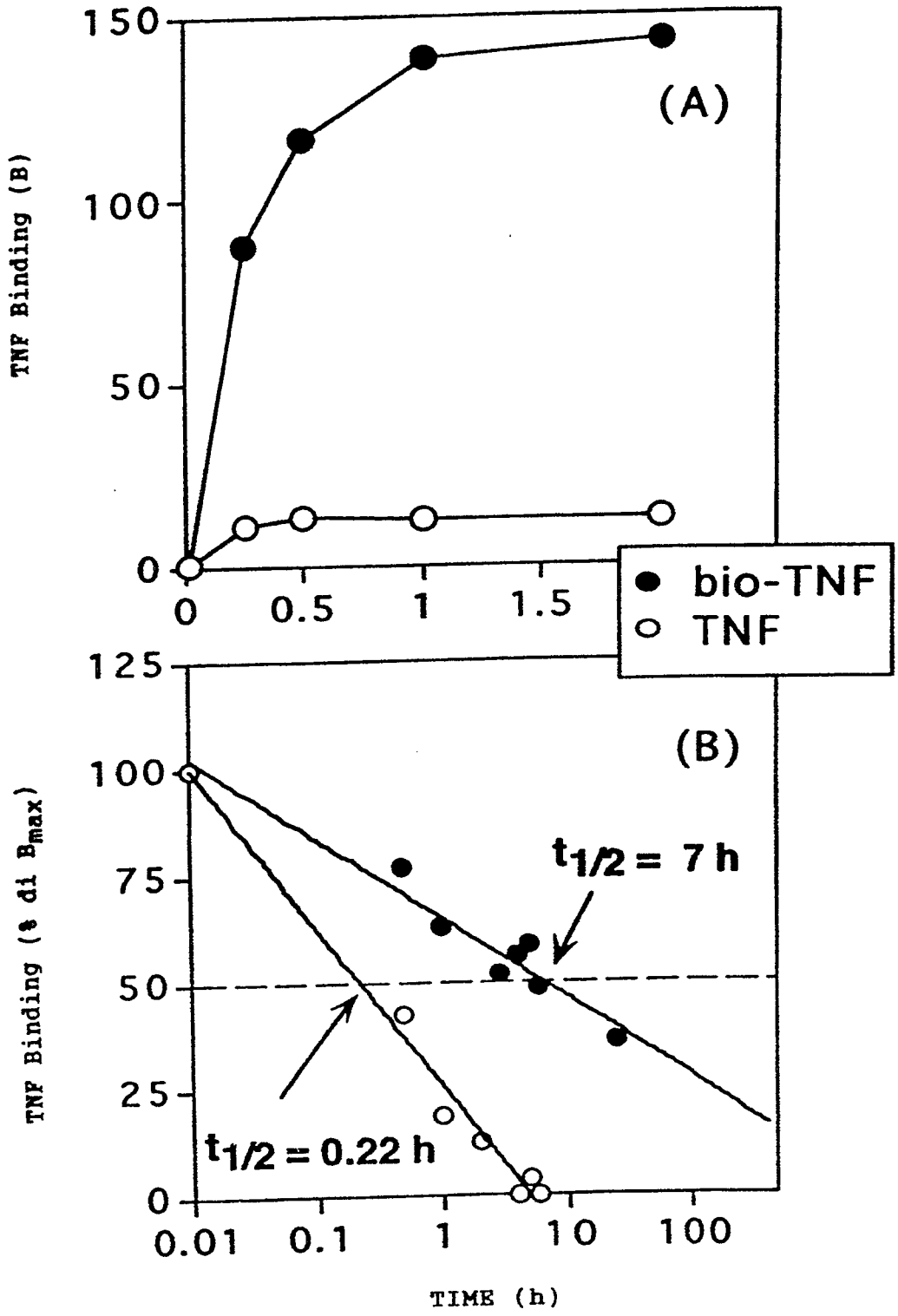
10. Tumoral necrosis factor according to claims 7 or 8, wherein a protein or a fragment of biotinylated protein is the ligand.

11. Process for the preparation of the tumoral necrosis factor of claims 7-10 which comprise the reaction between the tumoral necrosis factor with biotin-6-aminocaproyl-N-hydroxysuccinimide at a pH between 5.5 and 7.5.

12. Biotinylated tumoral necrosis factor obtainable by mixing the subunits from the biotinylated and the non-biotinylated tumoral necrosis factor in a ratio 1:3 for 24-72 h at 4°C.

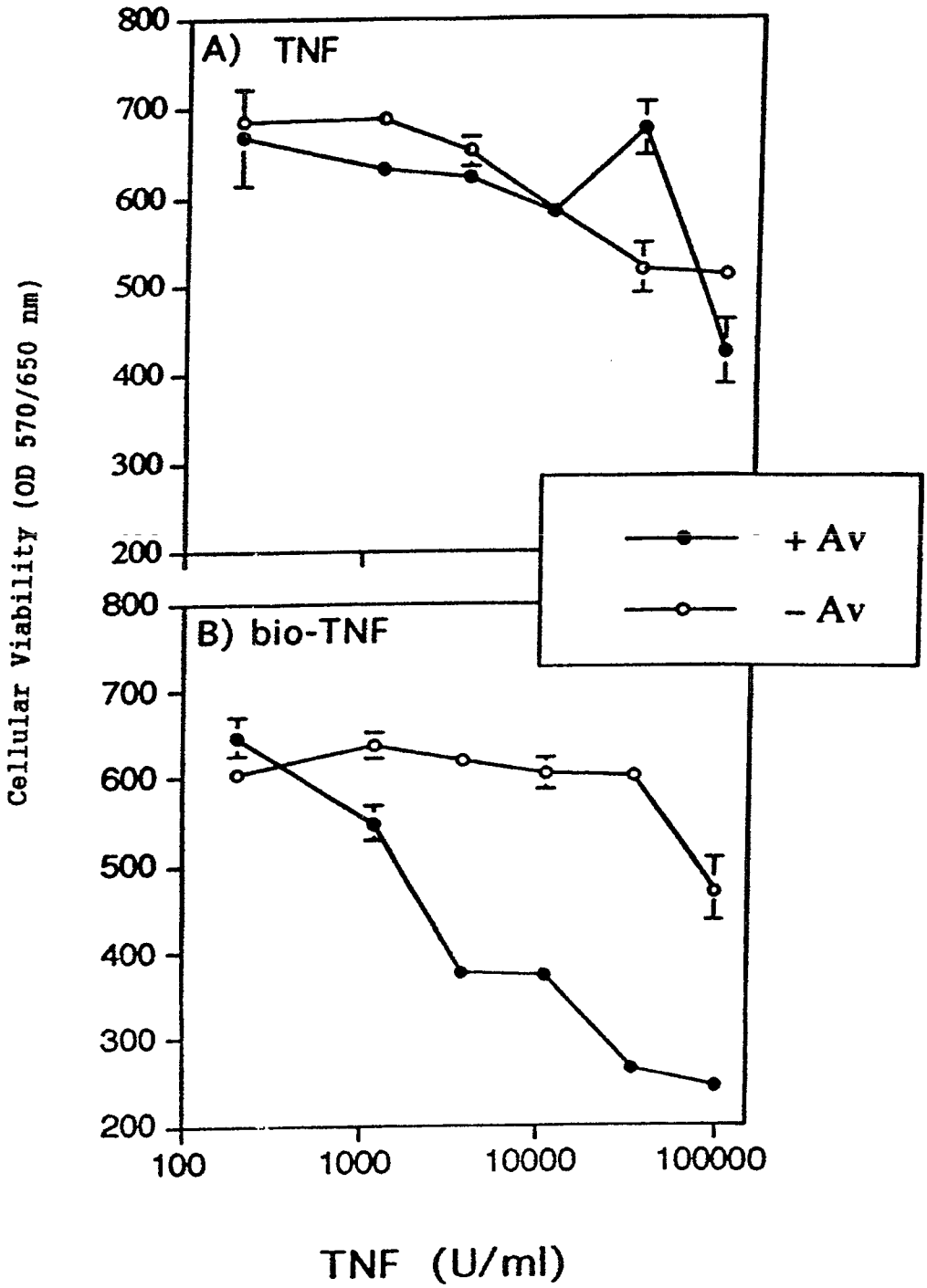
1/2

FIGURE 1


150
100
50
0
TNF Binding (B)
(A)
0
0.5
1
1.5
bio-TNF
TNF
125
100
75
50
25
0
TNF Binding (% di Bmax)
(B)
t1/2 = 7 h
t1/2 = 0.22 h
0.01
0.1
1
10
100
TIME (h)

2/2

FIGURE 2


A) TNF
B) bio-TNF
+ Av
– Av
Cellular Viability (OD 570/650 nm)
800
700
600
500
400
300
200
100
1000
10000
100000
TNF (U/ml)