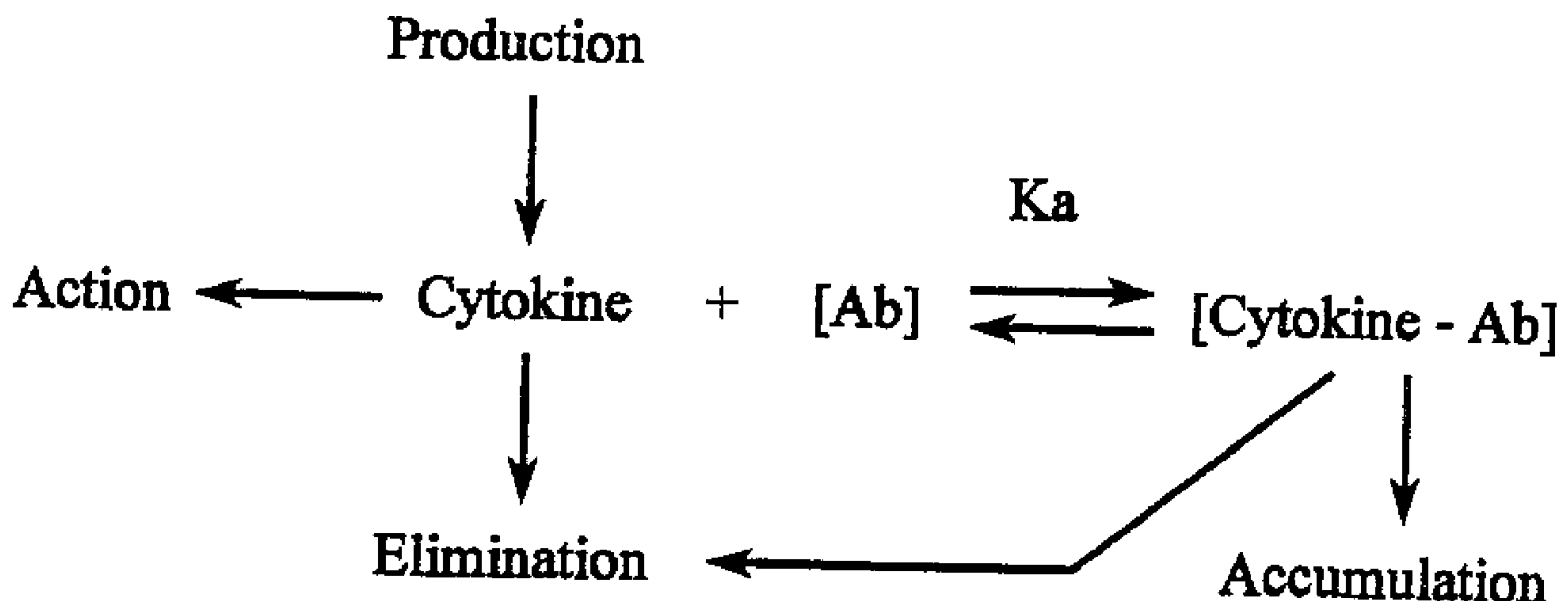




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The present invention concerns compositions for promoting the healing of wounds or fibrotic disorders comprising at least one agent specific to either a growth factor or a protein associated therewith in a system and which affects the quantity of active growth factor in the system, in combination with a pharmaceutically acceptable carrier, diluent or excipient.



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(21) International Application Number: PCT/GB95/00704 (22) International Filing Date: 29 March 1995 (29.03.95) (30) Priority Data: 9406154.6 29 March 1994 (29.03.94) GB 9424898.6 9 December 1994 (09.12.94) GB (71) Applicant (for all designated States except US): THE VICTORIA UNIVERSITY OF MANCHESTER [GB/GB]; Oxford Road, Manchester M13 9PT (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): FERGUSON, Mark, William, James [GB/GB]; Dept. of Cell & Structural Biology, University of Manchester, Coupland III Building, Manchester M13 9PT (GB). SHAH, Mamta [IN/GB]; 12 Lathom Road, Withington, Manchester M20 9NX (GB). (74) Agents: McNEIGHT, David, Leslie et al.; McNeight & Lawrence, Regent House, Heaton Lane, Stockport, Cheshire SK4 1BS (GB).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). 2186269 Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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WOUND HEALING

The present invention concerns compositions for promoting the healing of wounds or fibrotic disorders.

At present there is a lack of compositions for treating wounds or fibrotic disorders.

By "wounds or fibrotic disorders" is meant any condition which may result in the formation of scar tissue. In particular, this includes the healing of skin wounds, the repair of tendon damage, the healing of crush injuries, the healing of central nervous system (CNS) injuries, conditions which result in the formation of scar tissue in the CNS, scar tissue formation resulting from strokes, and tissue adhesion, for example, as a result of injury or surgery (this may apply to e.g. tendon healing and abdominal strictures and adhesions). Examples of fibrotic disorders include pulmonary fibrosis, glomerulonephritis and cirrhosis of the liver, in which TGF- β_1 has been implicated and proliferative vitreoretinopathy, in which TGF- β_2 has been implicated.

In particular, there is a lack of compositions for promoting the healing of wounds or fibrotic disorders with reduced scarring. Scar tissue formation, although providing mechanical strength to a healed wound, can be unsightly and may impair the function of the tissue.

This is particularly the case in wounds which result in scar tissue formation in the CNS, the scar tissue inhibiting the reconnection of severed or re-growing nerve ends, so significantly affecting their function.

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There is also a lack of compositions for use in the treatment of chronic wounds, for example venous ulcers, diabetic ulcers and bed sores (decubitus ulcers), especially in the elderly and wheel chair bound patients. Such compositions may be extremely useful in patients where wound healing is either slow or in whom the wound healing process has not yet started. Such compositions may be used to "kick-start" wound healing and may then be used in combination with compositions (e.g. those of PCT/GB93/00586) which promote the healing of wounds or fibrotic disorders with reduced scarring. Hence not only may a chronic wound be healed, but it may be healed with reduced scarring.

WO 92/17206 discloses compositions for use in the treatment of wounds to inhibit scar tissue formation during healing, comprising an effective activity-inhibiting amount of a growth factor neutralising agent or agents specific against only fibrotic growth factors together with a pharmaceutically acceptable carrier.

WO 93/19769 discloses a healing composition containing at least one non-fibrotic growth factor in combination with a pharmaceutically acceptable carrier.

These publications disclose the use of antibodies to TGF- β_1 , TGF- β_2 and PDGF; binding proteins which prevent TGF- β_1 , TGF- β_2 and PDGF from binding to their receptors by binding to the proteins or their receptors; soluble forms of the growth factor receptor or the growth factor binding domains of the receptors; and antisense oligonucleotides or ribosymes which act to prevent fibrotic growth factor mRNA translation.

Surprising results have now been obtained by the present inventors using

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antibodies specific to growth factors and proteins associated therewith.

According to the present invention there is provided a composition for promoting the healing of wounds or fibrotic disorders comprising at least one agent specific to either a growth factor or a protein associated therewith in a system and which affects the quantity of active growth factor in the system, in combination with a pharmaceutically acceptable carrier, diluent or excipient.

By "agent" is meant any compound or molecule which is capable of affecting the quantity of active growth factor in a system. Such an agent may work by neutralising, activating or deactivating a growth factor, affecting its half-life or by causing a change in its production level.

By "system" is meant the system to which the composition is applied and as such is defined by both the nature of the wound or fibrotic disorder and by the method of application of the composition. For example, in the case of a surface skin wound to which a composition is topically applied, "system" means the surface skin wound, and in a CNS disorder which is treated by injection of a composition into the blood stream, "system" means the body as a whole, whereas in a CNS disorder which is treated by a localised injection of a composition into the CNS, "system" means the CNS, in particular the area to which the injection was localised.

By "active growth factor" and "active quantity of growth factor" is meant growth factor which can effect a response in its target cells

At least one agent may comprise an antibody or a fragment or a derivative

thereof which binds to a growth factor or protein associated therewith.

Such an antibody may be selected from any one of the group of a monoclonal, phage, polyclonal and genetically engineered antibody.

At least one agent may comprise an antibody fragment comprising only the active binding site.

At least one agent may be specific to a single epitope.

At least one agent may be specific to a non-fibrotic growth factor; such a growth factor may be selected from the group of FGF-1, FGF-2, FGF-7, EGF, TGF α , IL-10, IL-12, IL-17, TGF- β_3 and IFN α .

At least one agent may be specific to a fibrotic growth factor; such a growth factor may be selected from the group of TGF- β_1 , TGF- β_2 , PDGFAA, PDGFBB, PDGFAB, a member of the CTGF family, IL-1, IL-2, IL-8 and TNF α .

A composition according to the present invention may promote the healing of chronic wounds or it may promote the healing of wounds with reduced scarring.

At least one agent may act to potentiate the quantity of an active growth factor. Hence the increased level of active endogenous growth factor so potentiated may act to promote the healing of a wound.

Such a composition according to the present invention may promote the

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healing of chronic wounds and comprise at least one agent which potentiates a fibrotic growth factor. It may comprise an antibody specific to a single epitope on a growth factor selected from either one of the group of TGF- β_1 and TGF- β_2 .

The inventors have found that, surprisingly, antibodies specific to a single epitope of TGF- β_1 and TGF- β_2 can either have no effect on the growth factor or can cause a potentiation if the growth factor.

Obviously not all antibodies specific to a growth factor will act to potentiate it since many may act to neutralise it or to inhibit its activity or may even have no noticeable effect. However, standard laboratory techniques may be used in order to generate and test antibodies and isolate those which act to potentiate growth factors.

Such a potentiation of a growth factor was unexpected, although potentiation has previously been observed in different systems. Heremans *et al.* (Eur. J. Immunol., 1992, 22: 2395-2401) and Martens *et al.* (Eur. J. Immunol., 1993, 23: 2026-2029) showed the potentiation of the cytokine IL-6 by the injection of monoclonal antibodies specific to IL-6 into mice which had previously been injected with endotoxin in order to induce a generalised Schwartzman reaction (endotoxic shock).

The exact mechanism of potentiation remains unknown. However, the hypotheses put forward by the research teams to explain these results may be applied to the present inventors own findings. For example, they propose that if the affinity of the cytokine receptor for the cytokine is higher than that of the antibody, the cytokine may still be active despite its binding to the antibody.

Alternatively, the increased cytokine levels after administration of antibody may be due to delayed elimination or to increased production. Delayed elimination of endogenous cytokine might be brought about by complex formation of the cytokine with the antibody. Such antibodies may act as carriers of cytokines, preventing their elimination from the circulation, urine etc. Alternatively, or additionally, a cytokine may exert negative control over its own synthesis and/or release *in vivo*. Thus partial neutralisation or sequestration of the cytokine at the tissue level may depress release, resulting in higher production levels (illustrated in Figure 1). Finally, low affinity antibodies to the active site of the growth factor (so called "neutralising antibodies") may be most effective as potentiating complexing agents as they protect the active site from degradation.

A composition according to the present invention may also comprise at least two agents each of which is specific to a single different epitope on a growth factor, such that individually the agents either have no effect or act to potentiate the active quantity of the growth factor, but which in combination act to reduce the active quantity of the growth factor, the reduced quantity of the active growth factor acting to promote the healing of a wound.

Such a composition may comprise two antibodies.

Such a composition may be specific to a growth factor selected from one of the group of TGF- β_1 and TGF- β_2 , the composition acting to promote the healing of a wound with reduced scarring.

Hence a composition according to the present invention may comprise two

antibodies each of which is specific to a single different epitope on TGF- β_1 , such that individually the antibodies either have no effect or act to potentiate the active quantity of TGF- β_1 , but which in combination act to reduce the active quantity of TGF- β_1 , the reduced quantity of the active growth factor acting to promote the healing of a wound.

Again the exact mechanism of this neutralisation is unknown. However, it is likely that recognition of two or more epitopes by two or more neutralising antibodies or fragments thereof results in the formation of a large enough antibody/growth factor complex for rapid detection and clearing by the body's systems (by kupfer cells of the liver), and hence neutralisation. By contrast the detection of a single epitope by a single antibody, or fragment thereof, may result in a small complex, not easily detected and hence either no effect or potentiation of the growth factor. Further, as summarised in Figures 2 and 3 the affinity of the antibody (low affinity for potentiation, high affinity for neutralisation) and ratio of antibody to growth factor (high for neutralisation, low for potentiation) etc. are likely to influence the end result. It follows that the same antibody may be used to achieve either neutralisation or potentiation according to circumstances and to whether it is coupled with another neutralising antibody recognising a different epitope. It further follows that genetically engineered antibodies or coupled antibodies or fragments thereof, e.g. diabodies capable of for example binding one or more epitope or fragments of larger complexes might also be used for the purposes of neutralisation.

A composition according to the present invention may comprise at least two agents specific to at least two growth factors, such that individually the agents either have no effect or have a slight neutralising effect upon the active quantity of their respective growth factors, but which in combination have a significantly greater

neutralising effect upon the active quantity of their respective growth factors.

Such a composition may comprise two antibodies.

Such a composition may comprise two antibodies, one being specific to a single epitope on TGF- β_1 and which has a slight neutralising effect upon the active quantity of TGF- β_1 , and the other being specific to a single epitope on TGF- β_2 and which has no effect upon TGF- β_2 , but which in combination act to reduce the active quantities of TGF- β_1 and TGF- β_2 , thereby promoting the healing of wounds with reduced scarring.

The inventors have found that, surprisingly, a composition comprising two antibodies, one being a neutralising antibody specific to TGF- β_1 and the other being an antibody specific to TGF- β_2 but which has no effect on it, does not in fact give the expected results. Instead of the composition having a slight neutralising effect upon TGF- β_1 and no effect upon TGF- β_2 , it was found that it actually had a significantly increased neutralising effect upon TGF- β_1 and TGF- β_2 , the two antibodies although specific to different proteins apparently acting synergistically.

The blocking and potentiating properties of neutralising antibodies to TGF- β_1 and TGF- β_2 are summarised in Figures 2 and 3.

Alternatively, a composition according to the present invention may comprise an agent specific to a protein associated with a growth factor and which reduces the active quantity of the growth factor associated therewith.

Such a composition may act to promote the healing of wounds with

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reduced scarring and comprise an agent specific to the Latency Associated Peptide associated with either the latent TGF- β_1 or latent TGF- β_2 complex.

The inventors have found that, surprisingly, an antibody specific to the latency associated peptide which forms a part of the latent TGF- β complex acts to affect a reduction in the quantity of active TGF- β_1 .

The exact nature of the mechanism by which this reduction in the active quantity of TGF- β_1 is affected remains unknown, although the binding of the large latent TGF- β complex to smooth muscle cells is blocked by the anti-LAP antibody (Sato *et al.*, 1993, J. of Cell Biol., 123: 1249-1254).

A composition according to the present invention may be used in conjunction with a composition which promotes the healing of wounds.

Such a composition which promotes the healing of wounds may promote the healing of wounds with reduced scarring.

Such a composition which promotes the healing of wounds with reduced scarring may comprise at least one non-fibrotic growth factor. At least one non-fibrotic growth factor may be selected from the group of FGF-1, FGF-2, FGF-7, EGF, TGF α , IL-10, IL-12, IL-17, TGF- β_3 and IFN α .

Thus the composition of the present invention may be used in combination with known compositions which promote the healing of wounds with reduced scarring. In particular, a composition according to the present invention which promotes the

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healing of chronic wounds may be used in a method whereby it is initially applied to the wound to "kick-start" wound healing, and then a composition which promotes the healing of wounds with reduced scarring may be used. Hence chronic wounds may not only be healed, but healed with reduced scarring

A composition according to the present invention may be used in a method of treatment or diagnosis of the human or animal body.

Also provided is a method of treatment of the human or animal body comprising the use of a composition according to the present invention.

The invention will be further apparent from the following description, with reference to the several figures of the accompanying drawings. Of the figures,

Figure 1 shows a possible effect of antibodies upon cytokine levels;

Figures 2 and 3 show the effect of antibody affinity and ratio of antibody to growth factor upon growth factor activity;

Figure 4 shows the rat experimental model used for the potentiation/neutralisation experiments;

Figure 5 shows the rat experimental model used for the Latency Associated peptide experiments.

Experimental Procedure

Potentialiation/Neutralisation of TGF- β_1 and TGF- β_2

Adult, male Sprague-Dawley rats (Charles River UK Ltd., Kent, UK) weighing 225-250 grammes were anaesthetised by halothane, nitrous oxide and oxygen inhalation. After locally clipping the fur, four full-thickness, linear incisions, 1cm in length, down to and including the panniculus carnosus were made on the dorsal skin of the animal. The incisions were placed equidistant from the midline and adjacent to the four limbs (Figure 4).

In each animal one of the wounds (control) was unmanipulated, one (sham-control) was injected with PBS/0.1%BSA/4mM HCl (carrier used to make up the TGF- β isoforms), one was injected with TGF- β_1 , TGF- β_2 or TGF- β_3 , and one was injected with neutralising antibody to TGF- β_1 alone, neutralising antibody to TGF- β_2 alone or a combination of neutralising antibodies to TGF- β_1 and TGF- β_2 (Table 1). The actual site of each treatment was rotated between the four wounds to control for anterior-posterior differences in the healing of rodent wounds. All injections were of 100 μ l each and administered by local infiltration of the wound margins (inset in Figure 4). The wounds were treated on days 0, 1 and 2 post-wounding. Animals were allowed to recover and housed in individual cages and fed normal rat chow and water ad libitum. Animals were killed by chloroform overdose on various days after wounding and the wounds harvested.

In order to determine the optimum doses of isoform specific neutralising antibodies to TGF- β_1 and TGF- β_2 , dose response experiments were performed. Animals

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were divided into two groups: in group I, one of the wounds was treated with neutralising antibody to TGF- β_1 and one of the wounds with TGF- β_1 ; in group II, one of the wounds was treated with neutralising antibody to TGF- β_2 and one of the wounds with TGF- β_2 .

The isoform specific neutralising antibodies and the TGF- β isoforms used in these experiments were a gift from Dr. A.B.Roberts (Lab. of Chemoprevention, NIH, Bethesda, USA). These antibodies have been well characterised and the specificities established (Roberts, A.B. *et al.*, 1990, Growth Factors, 3: 277-286; Danielpour *et al.*, In Growth Factors, 2: 61, 1989).

16 animals per group were studied; wounds were analysed on days 7 and 14 post-wounding in each set of experiments.

Table 1: doses of TGF- β isoforms and neutralising antibodies thereto.

Set	Group I		Group II		Group III	
	Anti-TGF- β_1 $\mu\text{l/injection}$	TGF- β_1 ng/inj	Anti-TGF- β_2 $\mu\text{l/inj}$	TGF- β_2 ng/inj	Anti-TGF- β_1 + Anti-TGF- β_2 $\mu\text{l/inj}$ (of each)	TGF- β_3 ng/inj
A	0.01	0.1	0.01	0.1	0.01	0.1
B	0.1	1	0.1	1	0.1	1.0
C	1	10	1	10	1.0	10.0
D	5	50	5	50	5.0	50.0

(1 μl of Anti-TGF- β_1 neutralises approximately 4ng of TGF- β_1 *in vitro*; 1 μl of Anti-TGF-

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β_2 neutralises approximately 6ng TGF- β_2 *in vitro*. TGF- β_1 and TGF- β_2 were extracted from porcine platelets; TGF- β_3 was recombinant TGF- β_3 expressed in NIH 3T3 cells.)

After harvesting, the wounds were bisected, one half being frozen and processed for immunochemistry and the other half being fixed in 4% paraformaldehyde and processed for paraffin embedding.

Results

The results of this study showed that the exogenous addition of neutralising antibody to TGF- β_1 plus neutralising antibody to TGF- β_2 to incisional, cutaneous wounds in adult rodents reduces scarring. Neutralising antibody to TGF- β_1 plus neutralising antibody to TGF- β_2 reduced the monocyte and macrophage profile, neovascularisation, fibronectin, collagen III and collagen I deposition in the early stages of wound healing and improved the architecture of the neodermis, thereby reducing scarring. Exogenous addition of neutralising antibody to TGF- β_1 resulted in only a marginal reduction in scarring. By contrast, exogenous addition of neutralising antibody to TGF- β_2 to wounds did not alter scarring. None of the wounds showed delayed healing.

Full results and discussion of these experiments are published in the PhD Thesis of Mamta Shah, Cutaneous Scarring: Modulation by Transforming Growth Factor-Beta and its Antagonists, University of Manchester, 1993, Chapter 5.

Latency Associated Peptide

Two adult, male Sprague-Dawley rats were anaesthetised and their fur

locally clipped to allow for four full-thickness linear incisions, 1cm in length to be made on the dorsal skins of two adult male Sprague-Dawley rats. The incisions were made to include the panniculus carnosus and were placed equidistant from the midline and adjacent to the four limbs.

In each of the animals, one of the wounds was injected with control antibody, one was injected with phosphate buffered saline (the carrier of the antibodies), one remained unmanipulated and one was injected with neutralising antibody to the Latency Associated Peptide (LAP) associated with the Large Latent TGF- β_1 complex. All injections were of 100 μ l each and were administered by local dermal infiltration of the wound margins. Animals were allowed to recover and housed in individual cages and fed normal rat feed and water *ad libitum*. Animals were killed by chloroform overdose on various days after wounding and the wounds harvested.

The first animal was treated with a dose of 50 μ g of antibody per wound, administered by a single injection at the time of wounding. This animal was killed seven days post-wounding and the wounds removed and processed for histological examination.

The second animal was treated with a dose of 100 μ g per wound of neutralising antibody. This animal was killed forty days post-wounding. Wounds were removed and processed for histological examination. Histological examination involved staining using connective tissue stains, e.g. mallory, to highlight collagen, orientation and organisation within the wound site.

Results

Histological examination of the first animal at seven days post-wounding revealed very few differences between wounds treated with the neutralising antibody and the control wounds. There was, however, limited evidence of reduced collagen in the wounds treated with the neutralising antibody.

Histological examination of the second animal revealed a marked improvement in wound quality (scarring) at forty days post-wounding in the wounds treated with 100µg of neutralising antibody. Staining showed that the collagen fibres in the wound were thicker and organised in a "basketweave" fashion more reminiscent of normal dermis. Indeed, one wound was extremely difficult to distinguish from the normal dermis in the papillary layers of the dermis, mild disorganisation only being noticed in the reticular layers of the dermis and the cut panniculus carnosus.

Hence antibodies specific to proteins associated with fibrotic growth factors, and particularly neutralising antibodies specific to the Latency Associated Peptide (LAP) associated with the Large Latent TGF- β_1 complex, are able to promote the healing of wounds with reduced scar tissue formation.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. Use of an antibody specific to a Latency Associated Peptide component of Latent TGF- β_1 or of Latent TGF- β_2 , in the manufacture of a medicament for promoting healing of wounds or fibrotic disorders with reduced scarring.
2. Use of an antibody specific to a Latency Associated Peptide component of Latent TGF- β_1 or of Latent TGF- β_2 , in the manufacture of a medicament for inhibiting fibrosis in a patient in need thereof.
3. A composition comprising an antibody specific to a Latency Associated Peptide component of Latent TGF- β_1 or of Latent TGF- β_2 , together with a pharmaceutically acceptable carrier, for promoting healing of wounds or fibrotic disorders with reduced scarring.
4. A composition comprising an antibody specific to a Latency Associated Peptide component of Latent TGF- β_1 or of Latent TGF- β_2 , together with a pharmaceutically acceptable carrier, for inhibiting fibrosis in a patient in need thereof.

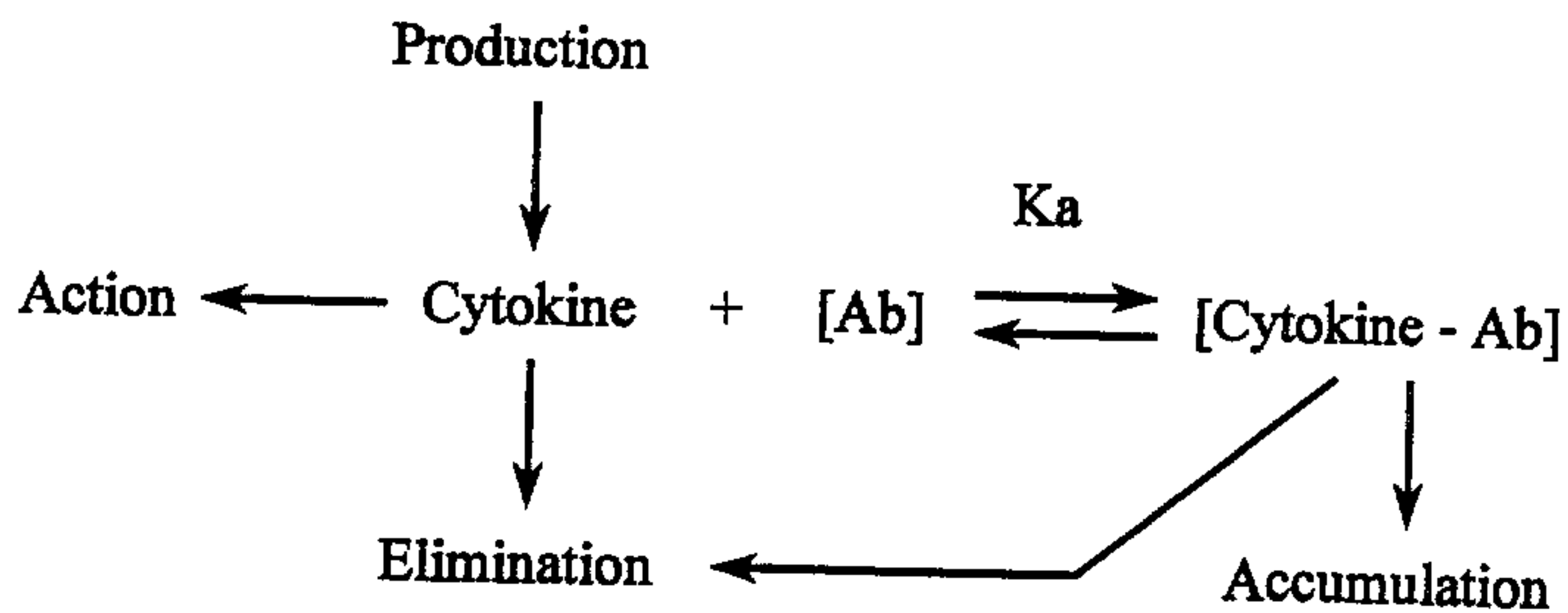
Figure 1

Figure 2

Effect	Production capacity	Affinity	Target/Assay Sensitivity	Clearance Rate	V _{on/off} of Establishing Equilibrium
Block	Low	High	Low	High	Rapid
Enhance	High	Low	High	Low	Slow

Figure 3Neutralising Antibodies to TGF- $\beta_{1,2}$

- 1) Anti-Scarring
 - Apply early - high ratio Ab/TGF- β
 - High affinity
 - Multiple epitope recognition

- 2) Enhance TGF- β activity e.g. to accelerate wound healing
 - Chaperone/bound reservoir
 - Apply late - low ratio Ab/TGF- β
 - Low affinity
 - Single epitope recognition

Figure 4

Experimental Model

A: Control wound
(unmanipulated)

B: Sham control
(PBS)

C: Experimental wound 1
(control antibody)

D: Experimental wound 2
(neutralising antibody)

Standard 1cm full thickness, linear
incision wound down to and including
the panniculus carnosus muscle.

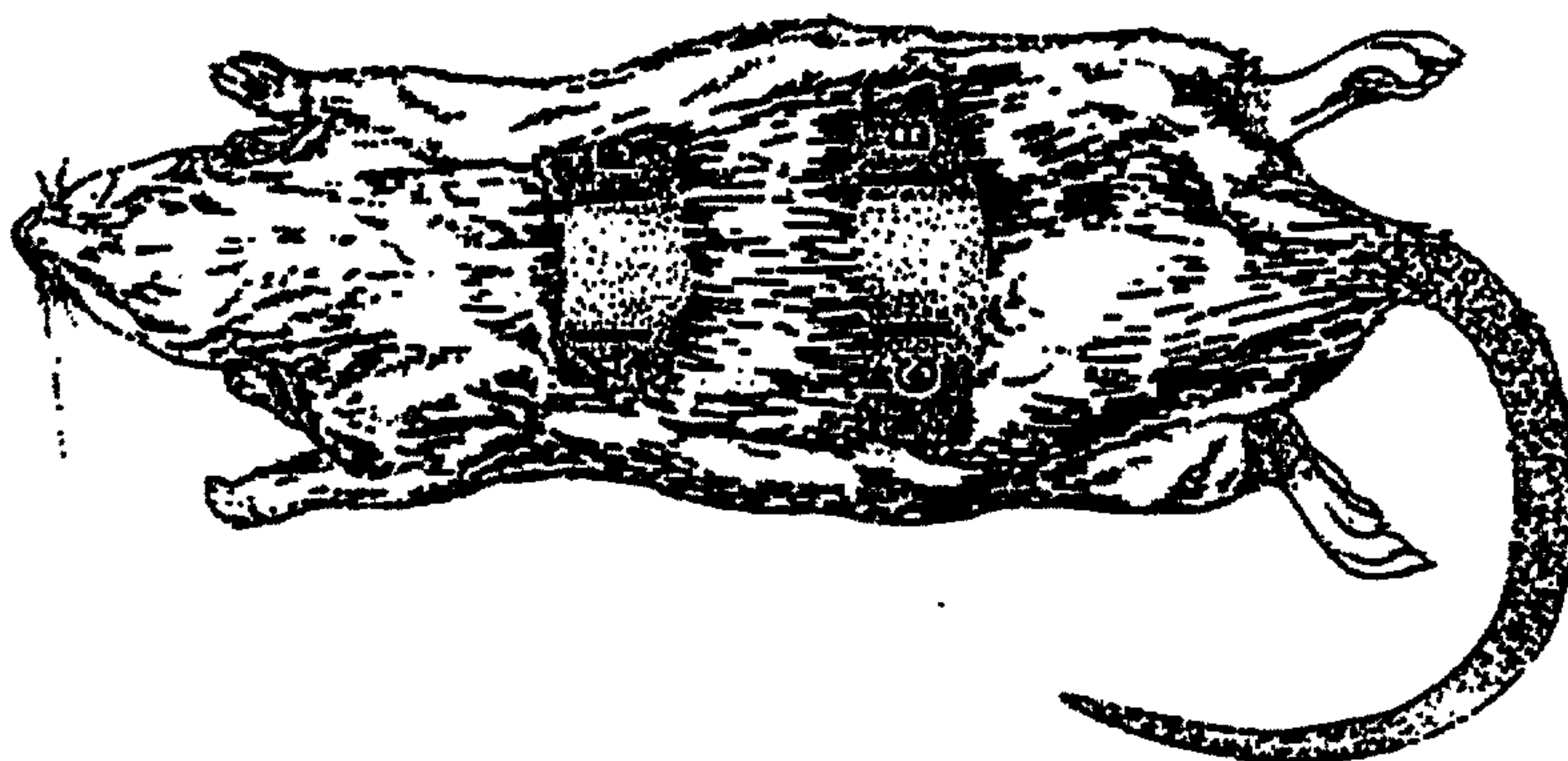
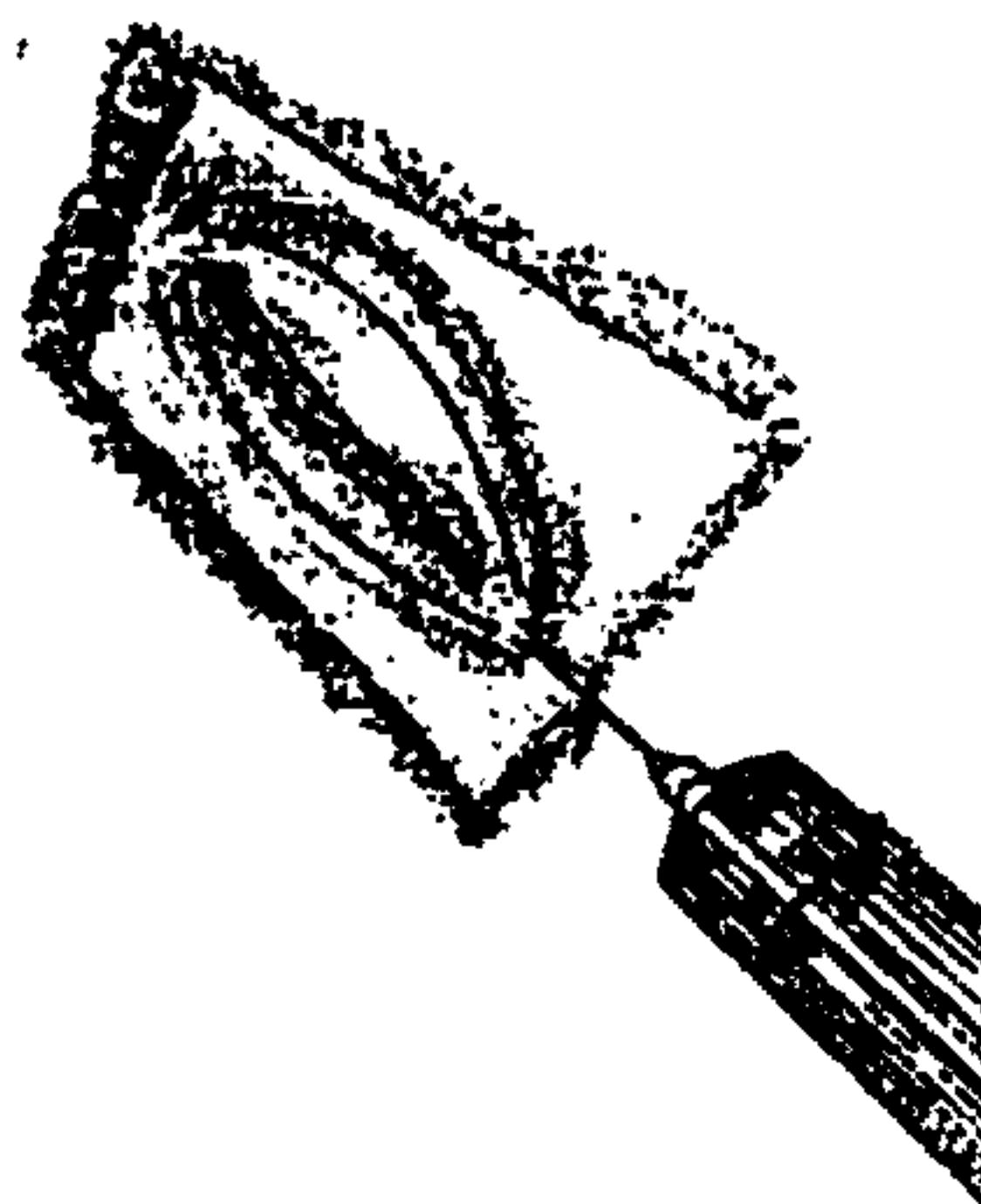


Figure 5 Experimental Model

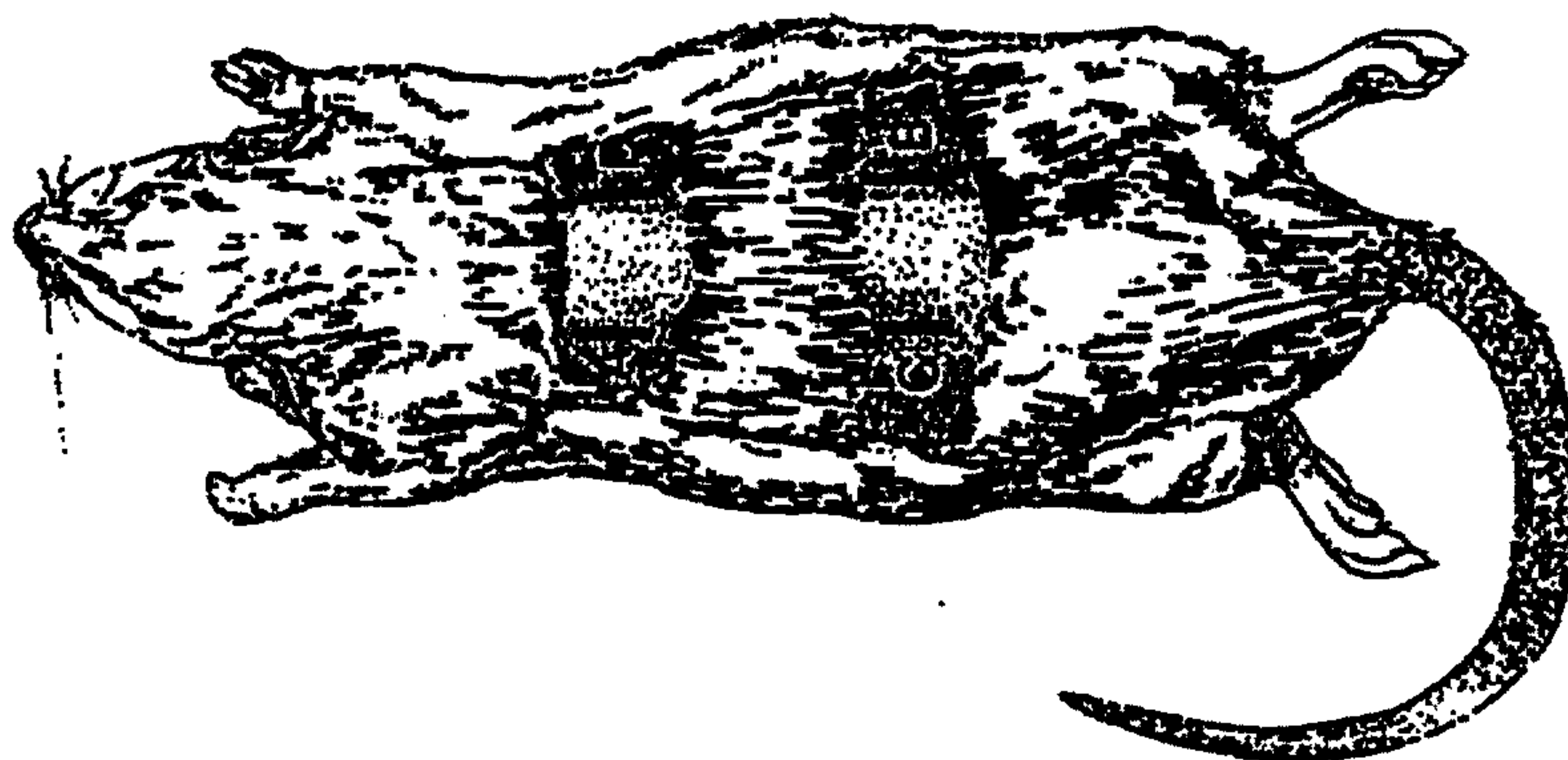
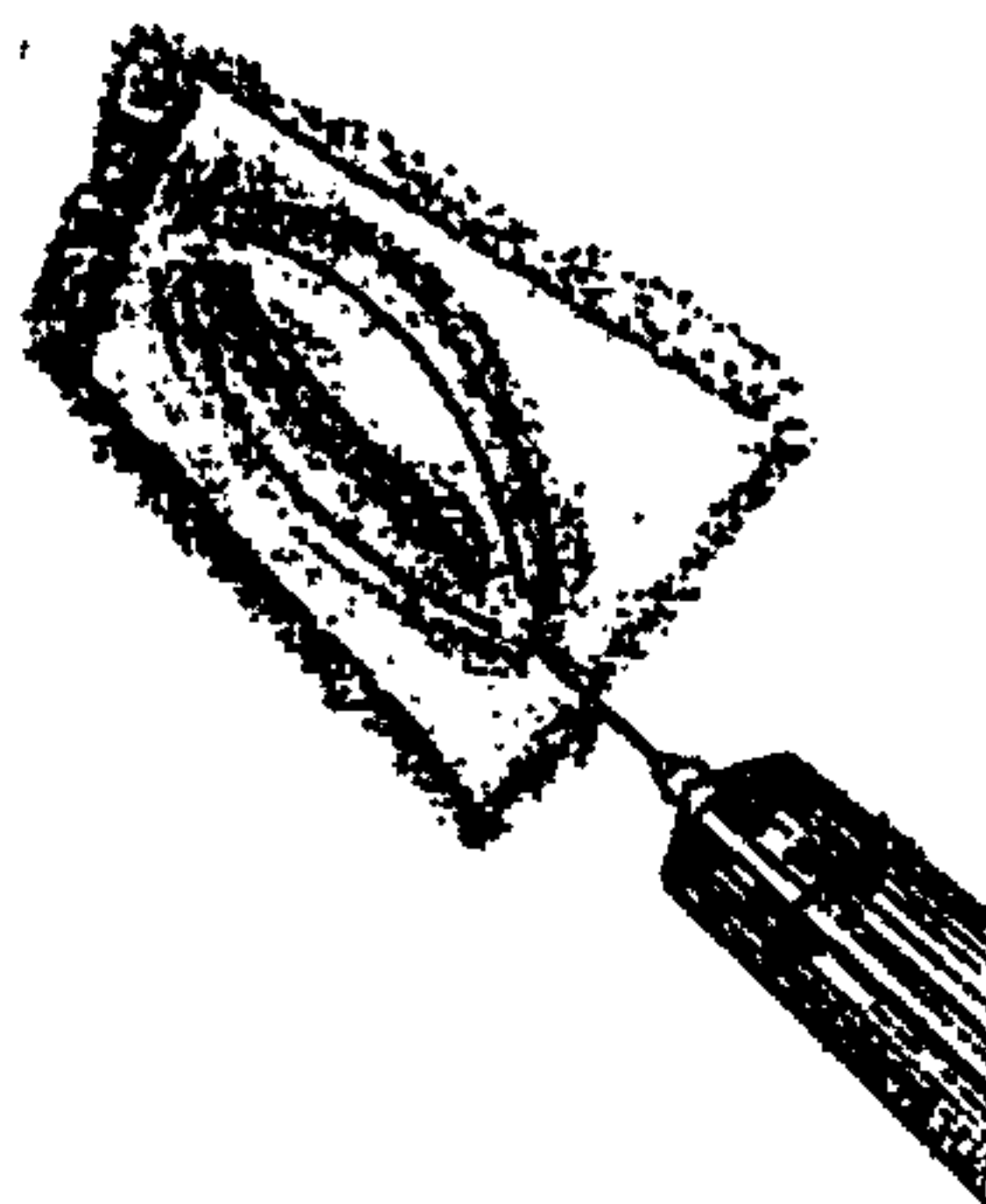
A: Control wound
(unmanipulated)

B: Sham control
(PBS/4mM HCl/0.1%
BSA)

C: Experimental wound 1
(Anti-TGF- β_1 or Anti-
TGF- β_2 or Anti-TGF- β_1 + Anti-
TGF- β_2)

D: Experimental wound 2
(TGF- β_1 or TGF- β_2)

Standard 1cm full thickness, linear
incision wound down to and including
the panniculus carnosus muscle.



Production



K_a

Action



Cytokine

+

[Ab]



[Cytokine - Ab]



Elimination



Accumulation

