The Effect of *In Vivo* T Helper and T Suppressor Lymphocyte Depletion on Wound Healing

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The role of T lymphocytes in wound healing is still not well-defined. Because it had been previously shown that *in vivo* depletion of T cells leads to impaired wound healing, the effect of depleting T cell subsets on subsequent fibroplasia was studied. T helper/effector cells were depleted by the use of the monoclonal antibody GK1.5, reactive against the L3T4 antigen (CD4). T suppressor/cytotoxic lymphocytes were depleted by using the 2.43 monoclonal antibody reactive against the Lyt 2 antigen (CD8). In the first experiment, Balb/c mice were treated with the antibodies starting at 24 hours before wounding was performed, and weekly thereafter. Depletion of the T helper/effector cells had no effect on wound-breaking strength or hydroxyproline deposition in sponge granulomas, whereas depletion of T suppressor/cytotoxic cells significantly enhanced both of these healing parameters. In a second experiment, T cell subset depletion was started on Days 0, 3, 7, 10, and 14 postwounding, and treatments were continued weekly thereafter. Once again, depletion of T helper/effector cells had no effect on wound healing, whereas depletion of T suppressor/cytotoxic cells markedly increased both wound-breaking strength and collagen synthesis. In conclusion, the data show that T suppressor/cytotoxic cells have a counter-regulatory role in wound healing, whereas the T cell subset responsible for up-regulating wound healing remains to be identified.

WITHIN THE LAST few years, it has become apparent that the T cell-dependent immune system plays an active role in the process of wound healing. It has been shown that abnormal fibroplasia can occur secondary to continued antigenic stimulation of host T lymphocytes.[1,2] We have previously shown that mice depleted *in vivo* of T lymphocytes (CD3) have impaired wound healing as assessed by both wound-breaking strength and reparative collagen synthesis.[3] However, the role of individual T cell subsets on fibroplasia is not known. In the present study, the effect of depleting T helper and T suppressor lymphocyte subsets by using specific monoclonal antibodies (MAB) were examined in order to analyze the role that individual T cell subsets play in wound healing.

Supported by NIH Grant # 1 R29 GM 38650-01.

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Submitted for publication: August 19, 1988.

Materials and Methods

Groups of ten male Balb/c mice (Harlan Sprague Dawley, Indianapolis, IN) 8–10 weeks old were used throughout the experiments. Groups of five mice were housed in clear plastic shoe box-type cages and were kept in a room with outside exposure at a constant temperature and humidity. Animals were fed a pellet diet (Teklad LM 485 Mouse/Rat Diet, Winfield, IA) that was nutritionally complete and were given tap water for drinking, both of which the mice were to take *ad libitum*. All mice were acclimatized to our laboratory conditions for at least 1 week before entry into the experiments. Mice were weighed at the start of the experiments and weekly thereafter.

Wounding Procedure

Under pentobarbital anesthesia (4 mg/kg BW intraperitoneal) the backs of mice were shaved and prepped with an organic iodine cleanser. A 2.5-cm midline dorsal skin incision was made sharply down to the level of the dorsal fascia. At the cephalad poles of the wound, subcutaneous pockets were created by use of scissors, and preweighed, sterile, moistened polyvinyl alcohol sponges (Unipoint Industries,) were inserted into these pockets. The wounds were then closed with a running 5-0 nylon suture.

Assessment of Wound-Healing

At 2, 3, and 4 weeks postwounding, respectively, the mice were killed. While the mice were under ether anesthesia, cardiac blood was obtained by open thoracotomy

TABLE 1. *Percentage Depletion of T Cell Subsets After Treatment with GK1.5 (Averages of 3–6 Separate Determinations)*

Site of Depletion	Time	Thy 1.2 (CD3)	L3T4 (CD4)	Lyt 2 (CD8)
Blood	24 hours	79.3%	79.8%	−81.8*
	2 weeks	62.6%	78.9%	−107.9%
	3 weeks	77.0%	70.1%	−119.5%
	4 weeks	65.4%	65.0%	−121.4%
Spleen	24 hours	66.9%	70.4%	−44.7%
	2 weeks	79.4%	62.8%	−101.1%
	3 weeks	69.8%	62.4%	−53.2%
	4 weeks	63.4%	57.2%	−124.0%

* A negative number implies an increase in percentage staining by the formula used.

technique into heparinized syringes. The spleen was also removed aseptically. Subsequently, the dorsal pelt containing the healing scar was removed and cut into 2–3 equal strips on a multibladed guillotine. Each strip was centered by a segment of the healing scar. The strips were placed in normal saline and were used within 60 minutes of harvest for assaying fresh wound-breaking strength, using a constant speed tensiometer. The implanted sponges were harvested, carefully freed of all surrounding fibrous tissue, and frozen at −20 C for subsequent determination of hydroxyproline content by the method of Woessner.[4] This is an assessment of the amount of reparative collagen synthesized at the wound site.

Generation of MABs

For the depletion of the T helper/effector subset, we used the GK1.5 MAB, which reacts against the L3T4 (CD4) antigenic determinant present on this subset.[5,6] The MAB 2.43, which reacts against an antigen present on T suppressor/cytotoxic (CD8) cells, was used for the depletion of this T cell subset.[7] Both of these rat anti-mouse MABs are of the IgG2b subtype, which makes them cytotoxic *in vivo*. Hybridomas secreting these antibodies were obtained commercially (American Type Culture Collection, Rockville, MD). Hybridoma cells were injected intraperitoneally into Pristane-primed nude balb/c mice. Ascites containing the MABs were obtained by tapping. The fluids were treated with 50% saturated ammonium sulfate, resuspended in PBS and extensively dialyzed against PBS. All MABs were stored frozen at −70 C. Antibody content was determined by assaying protein concentration and by immune electrophoresis. Control mice were injected with nonspecific rat immunoglobulin. MAB injections were administered intraperitoneally at a dose of $\approx$ 1 mg/mouse. Controls were given equivalent amounts of the rat serum immunoglobulin. Injections were started at various times in relation to the wounding time (see below) and were repeated at weekly intervals.

Determination of Efficacy of MAB Treatments

MAB treatment efficacy was assayed by determining the depletion of the respective T lymphocyte subset in both the peripheral circulation and spleen. Heparinized cardiac blood obtained from two to three mice at the time that the mice were killed was pooled, and blood mononuclear cells were separated by centrifugation over Lympholyte M (Cedar Lane Labs, Hornby, Ontario). Spleens from two to three animals were also pooled and minced, and the mononuclear cells were obtained after lysing the red blood cells with ammonium chloride (0.83%) and removal of macrophages by Petri dish adherence. Blood and spleen mononuclear cells were then stained with the following MABs: 1) FITC-labelled anti-Thy 1.2(CD3) against all T cells, 2) phycoerythrin-labelled GK1.5 (anti-L3T4; CD4) against helper/effector T cells, and 3) FITC-labelled anti-Lyt 2 (CD8) against T suppressor/cytotoxic cells (all from Beckton Dickinson, Mountain View, CA). Percentage staining was determined using a fluorescence-activated cell sorter (EPICS, Coulter Corp., Hialeah, FL). Data obtained from the fluorescence analysis were used to determine the percentage of T cell subset depletion in treated mice,[8] using the following formula:

$$\frac{U - D}{100 - D} = \frac{T}{100}$$

Where U = percentage of cells in untreated mice, T = percentage of cells in treated mice, and D = percentage of cells depleted from treated mice as a percentage of the values in untreated mice. The values for U and T were determined by fluorescence analysis and were used to calculate D, which is used to calculate the percentage of subset depletion according to the equation:

$$\%\text{depletion} = D/U \times 100$$

Experiment 1

In this experiment, lymphocyte subset depletion was begun 24 hours before wounding and weekly injections of MAB were administered. Mice were killed for wound-healing assessment at 2, 3, and 4 weeks postwounding for the GK1.5 treatment groups, and at 2 and 4 weeks postwounding for the 2.43 treatment groups. Groups of non-wounded animals were killed 24 hours after injections were made, in order to assess the lymphocyte depletion at the time of wounding.

Experiment 2

In this series of experiments, lymphocyte subset depletion was begun at various times postwounding (*i.e.*, Days 0, 3, 7, 10, and 14). After these initial injections, animals received repeated weekly treatments. All mice were killed 4 weeks after wounding for assessment of wound-healing.

TABLE 2. *Wound-Healing Parameters in Experiment 1 (OHP-hydroxyproline)*

	Breaking Strength (g + SEM)				OHP (μg + SEM)			
Time	Controls	GK1.5	2.43	p-value	Controls	GK1.5	2.43	p-value
2 weeks	212 ± 11	221 ± 12		NS	1263 ± 86	1318 ± 65		NS
3 weeks	287 ± 9	276 ± 12		NS	2295 ± 121	2278 ± 94		NS
4 weeks	341 ± 28	292 ± 16		NS	3189 ± 161	3051 ± 213		NS
2 weeks	222 ± 23		275 ± 14	<0.05	1340 ± 90		1773 + 7	<0.01
4 weeks	329 ± 27		408 ± 16	<0.02	3156 ± 162		4115 + 190	<0.001

Analysis of Data

All data are reported as mean ± SEM. Statistical analysis using the Student's t-test or analysis of variance was conducted using the StatView II program (Abacus Concepts, Berkeley, CA). Statistical significance was achieved at the 95% confidence level.

Results

In all experiments, weight gain of the animals was equal, regardless of the treatment regimens (data not shown). There were no macroscopic differences in the aspects of the wounds, and no infections were noted.

Experiment 1

Treatment with GK1.5 depleted the helper/effector T lymphocyte subset both in blood and in the spleen (Table 1). In the peripheral blood, depletions ranged from 79.8% (at 24 hours) to 65% (at 4 weeks). In the spleen, the depletions ranged from 70.4% to 57.2% at 24 hours and 4 weeks, respectively. There was also a similar depletion in the Thy 1.2 marker-bearing cells (all T cells). In parallel, there occurred a rise in the Lyt 2 subset (suppressor/cytotoxic). Depletion of helper/effector T cells with the GK1.5 MAB had no effect on wound healing when compared with that of control mice. Neither the wound-breaking strength nor the hydroxyproline content of the subcutaneously implanted sponges differed statistically between the two treatment groups (Table 2).

Treatment with the 2.43 MAB significantly depleted the suppressor/cytotoxic lymphocyte subset. In the peripheral blood, depletions ranged from from 93.4% at 24 hours to 94.1% at 4 weeks. In the spleen, these values ranged from 94.8% to 86.5% at 24 hours and 4 weeks, respectively (Table 3). As expected, there was a concomitant modest depletion in the Thy 1.2 cells and a smaller depletion in the number of L3T4 cells (Table 3). The 2.43-treated groups had markedly enhanced wound healing at 2 and 4 weeks postwounding (Table 2, Fig. 1). Highly statistically significant increases were noted in both the wound-breaking strength and the amount of hydroxyproline deposition in the implanted sponges.

Experiment 2

Once again, treatment with GK1.5, regardless of the time of starting the depletion in relationship to wounding, had no effect on any of the wound-healing parameters studied (data not shown). In terms of efficacy of depletion, treatments were uniformly successful in depleting the L3T4 subset with a concomitant depletion of the Thy 1.2 cells. The same rise in the percentage of suppressor/cytotoxic subset (Lyt 2) was observed (data not shown).

TABLE 3. *Percentage Depletion of T Cell Subsets After Treatment with 2.43 (Numbers Represent Averages of 3–6 Separate Determinations)*

Site of Depletion	Time	Thy 1.2 (CD3)	L3T4 (CD4)	Lyt 2 (CD8)
Blood	24 hours	19.6%	7.8%	93.4%
	2 weeks	14.5%	6.6%	84.2%
	4 weeks	8.9%	2.5%	94.1%
Spleen	24 hours	17.4%	13.6%	94.8%
	2 weeks	18.6%	11.2%	86.6%
	4 weeks	12.5%	5.4%	86.5%

Treatment with the 2.43 MAB significantly enhanced wound-healing, irrespective of the time that therapy was started in relation to the time of wounding (Table 4, Fig. 2). All wounds were studied at 4 weeks after wounding was performed, and all treatment groups (*i.e.*, those in which treatments were started on the day of wounding or on Days 3, 7, 10, and 14 after wounding) had significantly greater wound-breaking strength and collagen synthesis than controls.

Discussion

The data from these experiments demonstrate that T suppressor/cytotoxic cells have an inhibitory effect on

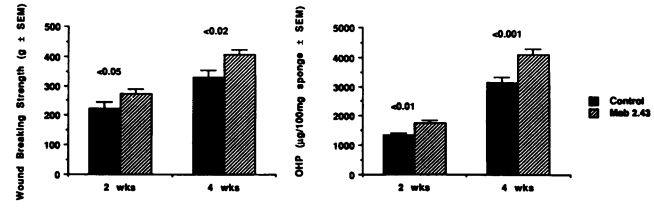

FIG. 1. Effect of T suppressor/cytotoxic lymphocyte depletion on wound-breaking strength and hydroxyproline (OHP) accumulation at 2 and 4 weeks postwounding. Note that MAB treatments were started 24 hours before wounding (data from experiment 1).

TABLE 4. *Effect of Treatment with 2.43 Starting at Various Times Postwounding on Wound-Healing Parameters at 4 Weeks (OHP-hydroxyproline)*

	Breaking Strength (g ± SEM)			OHP (μg/100 mg sponge ± SEM)		
Time	Controls	2.43	p-value	Controls	2.43	p-value
Day 0	410 ± 14	504 ± 16	<0.001	2440 ± 104	3073 ± 73	<0.001
Day 3	396 ± 17	496 ± 17	<0.001	2310 ± 54	2811 ± 140	<0.001
Day 7	443 ± 19	506 ± 19	<0.05	1906 ± 67	2477 ± 190	<0.006
Day 10	421 ± 15	565 ± 27	<0.001	2636 ± 105	3121 ± 118	<0.004
Day 14	407 ± 16	472 ± 18	<0.02	2613 ± 129	3247 ± 153	<0.004

wound healing. *In vivo* depletion of this subset, whether started before wounding was performed or as late as 14 days after wounding, leads to significant increases in wound-breaking strength and reparative collagen deposition, as assessed by the hydroxyproline content of the sponge granulomas. Conversely, depletion of T helper/effector cells by using the GK1.5 MAB had no effect on wound healing, regardless of when the depletion was started. This latter finding was somewhat unexpected. We had previously shown that depletion of T lymphocytes impairs wound healing and therefore expected that deletion of either the helper or suppressor T cell subset would duplicate these results. Furthermore, because depletion of T suppressor/cytotoxic cells enhanced wound healing, we would have expected that depletion of T helper/effector cells to lead to impaired healing. We hypothesize that because the depletion of T helper/effector cells was accompanied by a marked rise in the T suppressor/cytotoxic subset, this could have abrogated any effect that the use of GK1.5 may have had on wound healing. It should be noted that the rise in T suppressor/cytotoxic cells is relative, because the values are derived from the percentage staining. We did not determine absolute number of cells in either the peripheral blood or spleen.

There is ample evidence from previous *in vitro* and *in vivo* work that T cells participate in wound healing and have regulatory and counter-regulatory effects. T lymphocytes migrate into wounds following the influx of inflammatory cells and macrophages. The ratio of T helper to T suppressor lymphocytes at the wound site is greatly decreased in comparison to peripheral blood and lymph node subpopulation ratios.[9] Lymphokines, products of activated lymphocytes, have been shown to influence all cells that participate in wound healing. Transforming growth factor β (TGF-β) has been shown to be angiogenic *in vivo*.[10] TGF-β affects fibroblast activity directly by its potent chemotactic activity[11] or indirectly by inducing monocyte chemotaxis and secretion of fibroblast growth factors, including interleukin-1 (IL-1).[12] Once fibroblasts have been recruited and expanded, their synthesis of collagenous protein can be stimulated by lymphokines such as TGF-β[10] and lymphotoxin.[13] Fibroblast inhibitory lymphokines have also been described, most notably γ-interferon (IFN-γ), which inhibits fibroblast proliferation[14] and blocks fibroblast constitutive as well as growth factor-induced collagen synthesis.[15] It is evident that lymphokines can exert both stimulatory and inhibitory signals on all aspects of wound cellular activity and there seems to be a well-defined balance between these effects. Our knowledge is scant regarding how this balance is achieved; however, it is clear that an imbalance could result in wound failure or, conversely, in excessive fibrosis.

In conclusion, the present experiments clearly demonstrate that T suppressor/cytotoxic cells have a inhibitory effect on wound-healing. We are now further pursuing the identity of the T cell subset that upregulates wound healing, both under normal and pathologic conditions, such as excessive fibrosis.

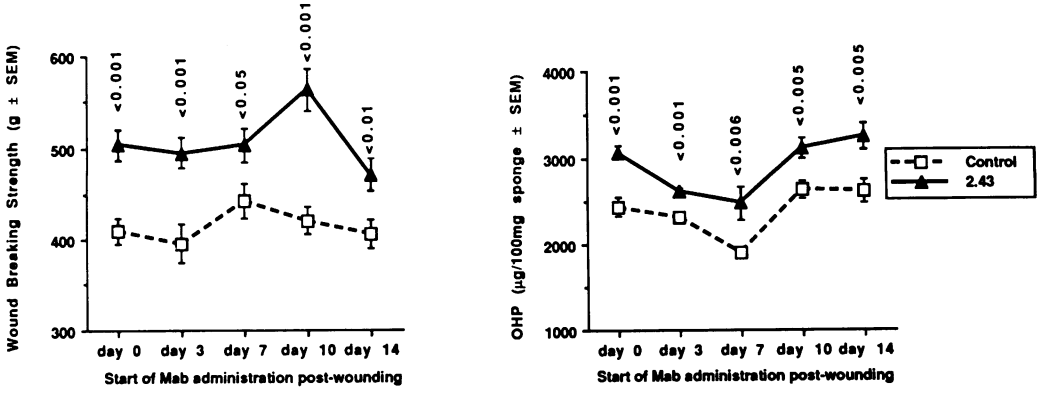

FIG. 2. Effect of T suppressor/cytotoxic cell depletion on wound-breaking strength and hydroxyproline (OHP) deposition at 4 weeks postwounding. Treatments with the MAB were started at various times postwounding, as indicated on the abscissa (data from experiment 2).

Acknowledgments

We thank Drs. J.A. Ledbetter and W.E. Seaman for their kind and helpful advise.

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