TUMOR NECROSIS FACTOR/CACHECTIN IS AN EFFECTOR OF SKIN AND GUT LESIONS OF THE ACUTE PHASE OF GRAFT-VS.-HOST DISEASE

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The introduction of allogeneic T lymphocytes into an immunoincompetent host is known to lead to a potentially lethal disease, the graft-vs.-host disease (GVHD), which is associated with lesions of various organs or tissues; in particular, in the acute phase of the disease, the intestinal tract and the epidermis (1–3) are affected. The mortality and tissue damage are clearly dependent upon the introduction of allogeneic T lymphocytes, but the proximal mechanisms of lesion formation have not been clarified. They might involve, for the acute GVHD, direct reactions between donor T lymphocytes and host target cells, or more indirect mechanisms mediated by interleukins released by the donor T cells (4, 5), thereby stimulating a variety of cells capable of inflicting tissue damage such as macrophages or large granular leukocytes (LGL) (6). There is experimental evidence that the chronic form of GVHD, which is characterized by different types of lesions, results from pathogenic mechanisms different from those involved in acute GVHD (7).

Tumor necrosis/cachectin (TNF-α), a protein produced mainly by macrophages, has a wide variety of actions in vivo and in vitro on many cell types other than malignant ones, and appears to be a major mediator in inflammatory processes (for review, see 8 and 9). The cutaneous and intestinal lesions characteristic of the acute phase of GVHD show, in addition to lymphocytic infiltration and epithelial alterations, isolated cell necrosis, epidermal cell necrosis in the cutaneous lesions (10), and necrosis of individual epithelial cells in the intestinal crypts (11). We therefore explored the possibility that TNF-α may play a role in the etiology of these lesions by treating mice undergoing acute GVHD with rabbit antibody against recombinant mouse TNF-α. This treatment almost entirely prevented the cutaneous and intestinal lesions of the acute-phase of GVHD, and markedly reduced overall mortality.

Materials and Methods

Mice. C57BL/10 (B10) and CBA/ca mice were purchased from Olac Ltd, Bicester, United Kingdom, and were bred for two to three generations in our animal facilities.

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1 Abbreviations used in this paper: BMC, bone marrow cells; GVHD, graft-vs.-host disease; LGL, large granular lymphocytes; LN, lymph node.

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C57BL/6 (B6) H-2^d mice were purchased from the Memorial Sloan-Kettering Cancer Center, New York.

**Induction of GVHD.** Recipient mice >3 mo old, were irradiated by a Cesium source (800 rad, delivered during 2–3 min) and injected intravenously with T depleted bone marrow cells (BMC), either supplemented with T lymphocytes prepared from lymph nodes (LN), or alone, as a control, according to a procedure described previously (4).

**Anti-rTNF-α Antibody.** IgG fraction was prepared by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) from the serum of a rabbit hyperimmunized with purified rTNF-α (12). The antibody activity of this IgG fraction was detected by immunodiffusion in agar against the immunizing antigen, and was quantitated in a TNF assay (13): a solution of 5 mg/ml neutralized the activity of 58 ng rTNF-α up to a dilution of 1:81,000. The antibody effect of this IgG fraction was also determined in vivo: a single intravenous or intraperitoneal injection of 2 mg completely prevented the cutaneous necrosis induced by a 7-d hypodermic perfusion of a solution of mouse TNF-α delivered by an osmotic minipump (Alza, Palo Alto, CA) (4 μg/d). Normal rabbit IgG, used as a control, was prepared by protein-Sepharose adsorption from the serum of nonimmunized rabbits.

**Histology.** Specimens were fixed in 2% formaldehyde/80% ethanol, embedded with parafin or methyl metacrylate, and 5- and 1-μm sections were stained with hematoxylin and eosin. For electron microscopy, specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer, 0.1 M pH 7.4; ultrathin sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Philips 400; Zurich, Switzerland).

**Results**

**Effect of Anti-TNF Treatment on GVHD-induced Mortality and Weight Loss.** Lethally irradiated (B10 x CBA)F1 mice were injected with T-depleted B10 BMC and, unless mentioned otherwise, 2 x 10^6 B10 LN cells as a source of parental T cells. Injection of this number of parental T cells leads to a GVHD that becomes clinically apparent (weight loss, hair ruffling) in about 2 wk, and is followed by a mortality of ~60% within 40 d, and >90% at 80 d (Fig. 1). To explore the possible effect of TNF on the course of this syndrome, mice were injected after 1 wk, i.e., before the beginning of the clinical symptoms, with 2 mg of rabbit anti-TNF IgG, or 2 mg of normal rabbit IgG as a control. This amount of anti-TNF was chosen because, in vivo, it entirely suppresses the effect of local administration of TNF (see Materials and Methods). After this first injection, mice were reinjected weekly with the same amounts of IgG until day
35; anti-TNF activity was detectable in the serum of treated mice until day 35 with titers ranging between 1:30-1:200. Anti-TNF treatment resulted in a decrease in mortality, with a survival rate of ~70% at days 40 and 80; normal rabbit IgG treatment had no effect on the course of the disease (Fig. 1). On day 18, normal rabbit IgG-injected GVHD mice showed a weight loss of 11% (±4%, mean ± SD) compared with control mice without GVHD (i.e., not injected with parental T cells), while there was no significant difference in weight between anti-TNF-injected GVHD mice and control mice. Somewhat comparable results of the anti-TNF treatment on mortality and weight loss were observed with a similar protocol in another strain combination involving only minor histocompatibility antigens (C57BL/6H-2k cells injected into CBA mice) (data not shown).

In spite of this effect of anti-TNF treatment, at no time during the course of the untreated GVHD was any TNF activity detectable in the serum. This is in contrast to what we have observed in mice with an acute malaria infection and neurologic symptoms, i.e., cerebral malaria, also prevented by anti-TNF treatment (14). On the basis of the course of the disease and the effects of anti-TNF treatment, the period of the 16–18th days was selected for histologic study of the skin and bowel.

**Passive Immunization with Anti-TNF IgG Prevents the Cutaneous Lesions of the GVHD.** The skin lesions observed in normal rabbit IgG treated hosts at the 16–18th day of the GVHD were similar to those previously reported in various donor-host combinations (15), and were characterized by: (a) an increase in the number of epidermal cell necroses (ECN); (b) foci of lichenoid hyperplastic reactions (LR); (c) areas of epidermal atrophy; (d) loss of the hypodermic fat. As shown in Fig. 2, these lesions, which are widespread enough to be all detected within a small skin area, were markedly decreased in anti-TNF-treated mice. Quantification of the various components of these lesions in the three groups of mice is shown in Table I. As can be seen by comparing Fig. 2, c and d, the anti-TNF treatment also markedly decreased lymphoid cell infiltration in the epidermis.

**Passive Immunization with Anti-TNF IgG Prevents the Intestinal Lesions of GVHD.** Mice with GVHD that were injected with normal rabbit IgG had severe intestinal lesions consisting of a dilatation and hypertrophy of the small bowel, marked flattening of the villi and elevation of the crypts, with an increased number of mitoses and frequent necroses of single cells within the crypt epithelium (Fig. 3, b and d). By electron microscopy, the single altered epithelial cells were characterized by condensation of the nuclear chromatin (Fig. 3 e) or marked compaction of the whole cell (Fig. 3 f), i.e., changes characteristic of the form of cell death called apoptosis (16, 17). In contrast to what has been observed in other models of intestinal GVHD studied at an earlier stage (days 5–6) (5), there was no marked infiltration of intraepithelial lymphocytes within the crypts; however, increased numbers of lymphoid cells, and occasionally neutrophils were present in the lamina propria. Electron microscopy showed that the endothelium of small venules was usually swollen. All these lesions were almost absent in the anti-TNF-treated mice (Fig. 2, a and c); the quantification of these changes is shown in Table I. Anti-TNF treatment also suppressed the increased expression
PIGUET ET AL.

Figure 2. Skin of mice on the 18th day of GVHD. Abdominal skin of mice treated with anti-TNF (A and C) or with normal rabbit IgG (B and D). At low magnification (original X 8), the anti-TNF–treated mouse (A) has a skin similar to that of control irradiated mice not injected with T lymphocytes (not shown), with a regular epidermis and presence of hypodermic fat (arrow); the control IgG-treated mouse (B) shows foci of epidermal atrophy (arrowhead) and lichenoid regeneration (thin arrows); the hypodermic fat is absent. At higher magnification (original X 400), the epidermis of the anti-TNF–treated mouse (C) has two to three cell layers with little or no cellular infiltration; in contrast, the epidermis of a control IgG-treated mouse (D) shows an area of lichenoid reaction with increment of the epidermal thickness, cellular infiltration (arrowhead) and necrosis of isolated epidermal cells (thin arrows).

Table I

Passive Immunization with Anti-TNF IgG Prevents the Cutaneous and Intestinal Lesions of the Acute Phase of the GVHR

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell injected</th>
<th>Treatment</th>
<th>Skin lesions</th>
<th>Intestinal lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ECN*</td>
<td>Atrophy and LRF†</td>
</tr>
<tr>
<td>a</td>
<td>BMC, anti-Thy-1</td>
<td>—</td>
<td>10.8 ± 8</td>
<td>1/6</td>
</tr>
<tr>
<td>b</td>
<td>BMC, anti-Thy-1</td>
<td>nIgG</td>
<td>64 ± 57</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ LN cells</td>
<td>23 ± 17</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>c</td>
<td>BMC, anti-Thy-1</td>
<td>Anti-TNF</td>
<td>23 ± 17</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Results are means ± SD of the values observed in six to nine individual mice in each group on the 16–18th day.

* Number of necrotic epidermal cells (ECN) by microscopic field (× 10²). Difference between group b and c: p < 0.05.
† Number of foci of epidermal atrophy and lichenoid reaction (LRF) per area of 2-cm histologic sections.
‡ Number of mice with detectable hypodermic fat per number of mice examined.
§ Difference between group b and c: p < 0.05.
∥ Measured with a calibrated ocular. X 400. p < 0.01 and <0.1.
** Number of crypt cell mitoses per microscopic field on thin sections (1 μ) examined at × 1,000 (p < 0.05).

of Ia on the gut epithelial cells observed in the GVHD lesions (data not shown) (5, 18).

Effect of Anti-TNF Treatment on the Spleen. On day 18, the spleens of GVHD mice were smaller than those of controls, with a recovery of 2.3 and 3.6 × 10⁷ cells for normal rabbit IgG– and anti-TNF–treated GVHD, respectively, compared with 8.6 × 10⁷ for control mice. The spleens of both anti-TNF- and normal IgG-treated mice showed structural disorganization, with growth of all
Table 11

**Anti-TNF IgG Does Not Prevent Foreign T Cell Proliferation in the Host Spleen**

PIGUE ET AL.

<table>
<thead>
<tr>
<th>LN cells injected (strain)</th>
<th>Treatment</th>
<th>Spleen</th>
<th>[(^{3})H]TdR incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CBA × B10)F₁</td>
<td>None</td>
<td>6 ± 2</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>B₁₀</td>
<td>nIgG</td>
<td>25 ± 4</td>
<td>598 ± 107</td>
</tr>
<tr>
<td>B₁₀</td>
<td>Anti-TNF</td>
<td>30 ± 9</td>
<td>515 ± 127</td>
</tr>
</tbody>
</table>

Results are the mean ± SD of the values of spleen cell [\(^{3}\)H]TdR incorporation observed in five (B₁₀ × CBA)F₁ host mice in each group, killed 5 d after lethal irradiation and transfer of 10⁶ syngeneic or B₁₀ LN cells. 5 × 10⁶ spleen cells were cultured for 4 h in the presence of [\(^{3}\)H]TdR, and the culture was then processed for scintillation counting (4).

Discussion

The present results show the TNF plays an essential role in the pathogenesis of the cutaneous and intestinal lesions of the acute phase of the GVHD. These lesions affect primarily the epithelial cells of the epidermis and of the gut mucosa, and usually appear during the second and third week after transplantation in man; in the mouse model used, the number of T lymphocytes added to the transplanted parental bone marrow cells was chosen to give a disease of comparable chronology and severity. The skin and gut lesions observed during the third week of GVHD were almost totally prevented by passive immunization with rabbit anti-TNF IgG; mortality during the following weeks was also mark-
edly reduced. In contrast, lesions of chronic GVHD that appear to have a different pathogenesis (7) were not modified; hepatic lesions were found in both groups after the fourth week of the disease (data not shown).

Two questions are addressed: what is the source of TNF during the acute phase of GVHD, and what is its possible mode of action? At no time during the course of the disease was TNF activity detectable in the blood, in contrast to what has been observed in mice with endotoxin shock (19) and with cerebral malaria (acute malaria complicated by neurologic symptoms) (14), two conditions that can also be prevented by anti-TNF treatment (14, 19). In the latter case, however, there is a marked accumulation of macrophages in the lymphoid organs (and in the brain vessels) (14), which is not observed in acute GVHD. The source of TNF in GVHD must therefore be local, in relation to the cellular infiltration of the skin and of the gut mucosa elicited by the response of donor lymphocytes to the host antigens. For the gut, the mechanisms initiating T cell infiltration of the whole intestinal mucosa have been established (5). The stimulated T cells release, among other lymphokines, IFN-γ (5), which is probably responsible for the increased Ia expression observed on the mucosal epithelia (5, 18), and may also result in an increased production of TNF-α by the local macrophages (19). TNF-α may also be released by the stimulated T lymphocytes themselves; TNF-α mRNA has indeed been detected in the gut intraepithelial T lymphocytes isolated from mouse GVHD (D. Guy-Grand, P. F. Piguet, and P. Vassalli, manuscript in preparation). Finally, another source of TNF-α may be the LGL, a cell variety that releases TNF-α upon stimulation (20), and which has been found in the skin lesions of mice with acute GVHD, where it is suspected to be responsible for the epidermal cell necrosis (6); proliferation of LGL may be induced by IL-2 released by stimulated, grafted T cells. Thus, there may be several local sources of TNF-α in the epidermal and intestinal lesions of acute GVHD. It is also possible that the rabbit anti-TNF-α antibodies recognize some crossreactive antigenic determinant on a product of activated lymphocytes that is structurally and functionally related to TNF-α, e.g., lymphotoxin or TNF-β (21), and that the effects observed in the present experiments are in part due to blockage of TNF-β. TNF-β has the same wide range of biologic activities as does TNF-α (7, 8). It should be noted, however, that rabbit anti-human TNF-α and -β antisera show no crossreactivity (22).

Besides its capacity to induce the necrosis of some types of tumor cells, TNF-α has a variety of effects on many cell types (8, 9), including necrosis of normal cells when injected in vivo in large amounts (23). It acts on cells involved in inflammation, in particular on endothelial cells, polymorphonuclear leukocytes, and monocytes; it leads to an increased adhesion of leukocytes to endothelial cell monolayers (24, 25), probably due to the increased expression, on endothelial cell membranes, of molecules associated with leukocyte and lymphocyte adhesion (26). It is thus likely that a local release of this mediator will lead to a self-aggravating chain of events, increasing the local concentration of inflammatory cells; this probably explains why the cell infiltration of the skin and gut lamina propria is so markedly decreased by anti-TNF treatment, while this treatment does not alter the stimulation of division of the foreign T lymphocytes by the host (Table II). The characteristic alterations of the acute
GVHD in the skin and the gut, however, are those of the epithelial cells. They consist of both isolated necrosis (within the rete ridge of the epidermis, and, in the gut, in the bottom of the crypts) and acceleration of epithelial renewal, manifested in the skin by the lichenoid hyperplastic reactions in the skin and in the gut by crypt hyperplasia with an increased number of mitoses. Because these alterations are so strikingly prevented by anti-TNF treatment, it appears that TNF acts on the less differentiated cells of the epithelium (i.e., the stem cells) in such a way as to induce their occasional death by apoptosis (16, 17) (as is also the case for malignant cells sensitive to TNF [17]) and their stimulation to divide. The hyperplasia might be a reparative process triggered by cell damage or death. However, TNF has also been shown to increase the responsiveness of cells to epidermal growth factor (EGF) (9); such an effect may therefore play a role in the intestinal lesions and in the lichenoid reactions in the skin, since it is known that epidermal growth factor accelerates in vivo skin (27) and gut epithelial renewal (28). Finally, in the skin, the striking disappearance of the hypodermic fat that accompanies the epidermal lesions is compatible with a local cachectin effect, related to the inhibitory action of TNF on the adipocyte lipoprotein lipase (8).

In conclusion, these experiments show that neutralization of TNF prevents the development of the cutaneous and intestinal lesions characteristic of the acute phase of GVHD. The favored interpretation is that the presence of activated lymphocytes within the skin and the gut mucosa results in an increment in the local production of TNF, which induces epithelial cell alterations and necroses, as well as an increase of the inflammatory reactions within these mucosae.

Summary

Lethally irradiated mice were injected with semiallogeneic, T-depleted bone marrow cells and an amount of peripheral T lymphocytes sufficient to induce graft-vs.-host disease (GVHD) becoming apparent on the second week after the graft and leading to an increasing mortality rate within the following weeks (>90% mortality within 80 d). Mice receiving bone marrow cells alone had no GVHD and were used as controls. Beginning on day 8, mice with GVHD were injected weekly with 2 mg of either rabbit anti-mouse recombinant tumor necrosis factor/cachectin (TNF-α) IgG, or normal rabbit IgG. On the 16–18th d, mice were killed to examine the skin and intestinal lesions of the acute phase of GVHD. The anti-TNF treatment resulted in an almost complete prevention of the severe lesions seen in the mice treated with normal rabbit IgG, i.e., the skin epidermal cell necrosis, foci of lichenoid hyperplastic reactions, and loss of the hypodermic fat; in the gut dilatation with marked flattening of the villi and elevation of the crypts, with increased numbers of mitoses and isolated crypt cell necrosis. In addition to preventing these acute lesions, anti-TNF treatment resulted in a significantly decreased mortality (~70% survival at 80 d). These results suggest that during acute GVHD, the activation of grafted lymphocytes leads to a local release of TNF in the cutaneous and intestinal mucosae, which induces epithelial cell alterations and increases the inflammatory reaction.
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References


