Acquired immunological tolerance of foreign cells is impaired by recombinant interleukin 2 or vitamin A acetate

(neonatally induced transplantation tolerance/T-cell growth factor/retinoid)

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Contributed by Peter B. Medawar, September 10, 1984

ABSTRACT The susceptibility of newborn mice to the inception of tolerance after exposure to antigen is associated with their deficiency in the production of endogenous interleukin 2 (IL-2). As further evidence of the complicity of IL-2 in the inception and maintenance of tolerance, it is shown here that a solid and long-lasting state of tolerance induced by the intravenous injection into newborn CBA mice of lymphoid cells from (CBA × C57BL/10ScSn)F$_1$ hybrids can be brought to an end by the administration of exogenous IL-2 or by supplementing an otherwise normal diet with vitamin A acetate, the effect of which is to increase the proportion of the moiety of the T-cell population that produces IL-2. These results indicate that certain nonspecific stimuli can influence whether immunological tolerance is maintained.

The immune system of vertebrates is normally unable to respond to potentially antigenic molecules encountered early in ontogenesis (1–4). This nonreactivity of the immune system toward various antigens, known as immunological tolerance, can also be induced in adult individuals (5, 6) and it can be explained in terms of (*i*) dominant suppression—i.e., the activity of suppressor cells capable of inhibiting immunocompetent cells involved in putting the positive immune response into effect (see e.g., ref. 7); (*ii*) failure in antigen presentation—i.e., the inability of antigen-presenting cells to provide sufficient specific information and/or additional nonspecific signals to initiate, support, or sustain the reactivity of antigen-specific lymphocyte clones (see e.g., ref. 8); or (*iii*) functional clonal deletion—i.e., the absence of any functional cells in the T- (or B-) cell repertoire specific for the antigen (see e.g., ref. 9). Since newborn mice are deficient in interleukin 2 (IL-2) (T-cell growth factor) production (10) and the susceptibility of newborn mice to the induction of tolerance is diminished by exogenous IL-2 (11), the inception of immunological tolerance could be interpreted as a consequence of reduced availability or effectiveness of IL-2 during the exposure of immunocompetent cells to antigens (12). Here we report that CBA mice made tolerant to C57BL/10ScSn (B10) alloantigens acquire responsiveness to these alloantigens when treated with recombinant IL-2 or vitamin A acetate (VAOAc).

MATERIALS AND METHODS

Mice and Their Diet. CBA, B10, and (CBA × B10)F$_1$ mice were obtained from the breeding colony of the Clinical Research Centre. The supplementation of diet of some mice with VAOAc (0.5 g per 1 kg of the conventional diet) was carried out as described (11, 13–18).

Induction of Tolerance to Alloantigens and Its Testing by Skin Transplantation. Bone marrow and spleen cells were obtained from 2- to 3-month old (CBA × B10)F$_1$ female mice. After washing in balanced salt solution supplemented with 2% heat-inactivated fetal calf serum, the cells were counted, pooled, pelleted at 200 × *g* for 7 min and resuspended to a concentration of 3 × 10^8 viable nucleated cells per ml. Newborn (<24 hr old) CBA mice received an intravenous injection of 0.05 ml (15 × 10^6 cells) via the anterior facial vein or sigmoid sinus (19). Ten- to 12-wk-old tolerized CBA mice were grafted with tail skin from B10 females (20). Grafts with irreversible necrotic changes over their whole area were considered rejected.

Preparation, Purification, Assaying, and Administration of Recombinant IL-2. The IL-2 gene was isolated from a cDNA library constructed from the mRNA of lectin-activated human splenocytes as described by Devos *et al.* (21). The *Escherichia coli* expression system has also been described by Devos *et al.* (21). The cells were grown in 101 fermenters and were harvested in stationary phase. After breaking the cells by release of gas pressure (22), the recombinant IL-2 was purified by reversed-phase high performance liquid chromatography (HPLC) (23), and the protein content of samples was estimated by the method of Lowry *et al.* (24). The proteins present before and after HPLC were analyzed by electrophoresis on polyacrylamide gels in the presence of NaDodSO$_4$, using the method of Laemmli (25). Protein molecular size markers of 14,400–92,500 daltons (NaDodSO$_4$/PAGE standards; Bio-Rad) were used. Separated proteins were visualized by Coomassie blue R250 staining (26). The specific IL-2 activity after purification was ≈6 × 10^6 units per mg of protein. The units of IL-2 activity were determined in a standard bioassay (27, 28) estimating the incorporation of [^{3}H]thymidine into indicator T lymphocytes. One unit of IL-2 activity, using CTLD indicator cells, was as defined in refs. 11 and 29. (The CTLD cell line, whose proliferation *in vitro* is strictly dependent on the presence of exogenous IL-2, was kindly supplied by Peter Lonai, Rehovot, Israel.) IL-2 was stored (1 mg·ml^{-1}) in 25 mM acetic acid at −80°C. Human albumin (fraction V; Miles) was added to a solution of IL-2 in balanced salt solution as a source of carrier protein to ensure stability of IL-2 throughout manipulation before *in vivo* use. The final concentration of human albumin was 1 mg·ml^{-1} and IL-2 concentration was 12 × 10^3 units·ml^{-1}. One hundred microliters of such a solution of IL-2 (1200 units) was injected intraperitoneally into tolerant mice 58 and 59 days after transplantation. As a control for stress, some tolerant mice received intraperitoneal injections of 100 μl of balanced salt solution containing human albumin (1 mg·ml^{-1}) at the same time.

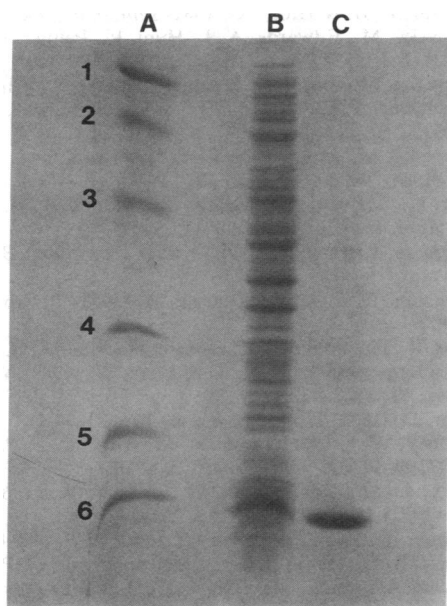Abbreviations: IL-2, interleukin 2; VAOAc, vitamin A acetate.

FIG. 1. Separation of human recombinant IL-2 from *E. coli.* Lanes: A, molecular weight markers (1, phosphorylase B, M_r 92,500; 2, bovine serum albumin, M_r 66,200; 3, ovalbumin, M_r 45,000; 4, carbonic anhydrase, M_r 31,000; 5, soybean trypsin inhibitor, M_r 21,500; 6, lysozyme, M_r 14,400); B, crude extract of *E. coli* after cell breakage; C, purified recombinant IL-2 after HPLC. Lanes A–C were loaded with 5, 30, and 3 µg of protein, respectively.

Statistical Methods. Times of skin allograft survival in the treatment groups were compared using logrank tests (30).

RESULTS

We decided to ascertain whether an already fully established state of transplantation tolerance could be influenced by immunomodulators such as IL-2 or VAOAc. In the present set of experiments, we induced long-lasting transplantation tolerance to alloantigens in newborn mice by an intravenous injection of semiallogeneic cells rather than a temporary state of unresponsiveness by an intraperitoneal injection as we did previously (11). Despite intravenous treatment with semiallogeneic cells, ≈60% of the CBA females and ≈40% of the CBA males rejected or started to reject their allografts within 58 days after transplantation. The mice that rejected their allografts or carried allografts showing signs of incipient necrosis were discarded from the present study. The rest of the mice, carrying healthy grafts, were randomly divided into three groups, each comprising 14 males and 12 females. Fifty-eight days after transplantation, we started to supplement the conventional diet of one group of tolerant mice with VAOAc. On the same day, the second group of tolerant mice received an intraperitoneal injection of 1200 units of recombinant IL-2 (Fig. 1), and the same amount of recombinant IL-2 was injected on the next day. The third group of mice
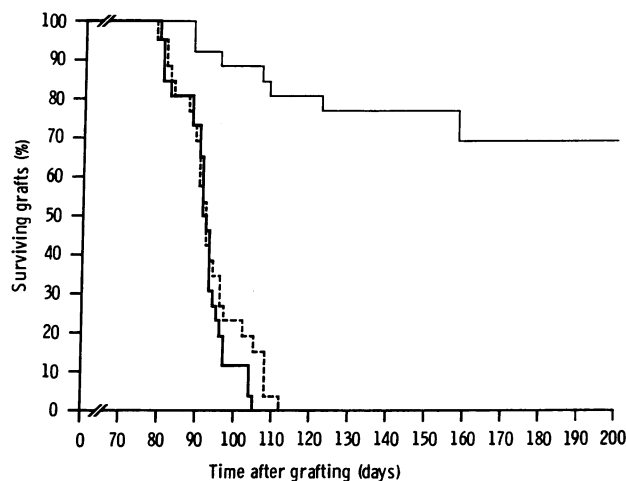

FIG. 2. Accelerated rejection of allografts in mice treated with IL-2 or VAOAc. CBA mice neonatally tolerant to B10 alloantigens and carrying B10 skin allografts were treated with recombinant IL-2 or VAOAc. Survival times of skin allografts were recorded. Abscissa indicates survival time of skin allografts. Ordinate indicates percentage of surviving allografts. Thin line, control group; thick line, group of mice treated with recombinant IL-2; dashed line, group of mice treated with VAOAc.

served as a control. Seven males and 6 females from this group received two control injections, and the same number of mice received no treatment. As expected, the control treatment did not affect the survival of skin grafts and therefore the data from both halves of the control group were pooled. All tolerant mice treated with recombinant IL-2 or VAOAc rejected their skin grafts in the period from 78 to 112 days after transplantation (Fig. 2; Table 1).

We performed logrank tests to compare the groups. The overall test, comparing all three groups, revealed highly significant differences between them ($P < 0.0001$). There was no evidence of a significant difference between the group of mice injected with recombinant IL-2 and the group of VAOAc-fed mice ($P = 0.8$). The control group was significantly different from the two treated groups ($P < 0.0001$). We also repeated the logrank tests adjusting for differences between male and female mice, but this did not affect the conclusions, as overall there were no significant differences in rejection times between males and females ($P = 0.6$).

DISCUSSION

It is beyond controversy that IL-2 influences T cells (31–33) and that vitamin A and its derivatives (retinoids) affect several aspects of the immune response (34, 35). In fact, it is possible that immunopotentiation by vitamin A is mediated through IL-2, because retinoids both increase IL-2 production (Gunther Dennert, personal communication) and induce a higher frequency of IL-2-producing cells in mice, as assessed by a limiting dilution analysis (unpublished observa-

Table 1. Influence of IL-2 or VAOAc on B10 allograft survival in tolerant CBA mice

Group	Females*			Males†			Total‡		
	Number of rejected grafts (%)	Rejection time, days		Number of rejected grafts (%)	Rejection time, days		Number of rejected grafts (%)	Rejection time, days	
		Median	Range		Median	Range		Median	Range
Control	5 (41.7)	>200	95->200	3 (21.4)	>200	88->200	8 (30.8)	>200	88->200
Recombinant IL-2	12 (100)	91	79–104	14 (100)	93	83–105	26 (100)	91.5	79–105
VAOAc	12 (100)	91	78–108	14 (100)	92	81–112	26 (100)	92	78–112

*$n = 12$.
†$n = 14$.
‡$n = 26$.

tions). It is pertinent to mention in this context that the Lyt-1^+2^- lymphocyte subset (proportionally increased in VAOAc-fed mice; see ref. 15) appears to be the main T-cell subpopulation mediating antiallograft responses (15, 36, 37) and producing IL-2 (38). These findings, taken together with the present ones, suggest that IL-2 could be involved in the initiation, development, and, perhaps, maintenance of the faculty of rejecting foreign cells and hence that manipulating IL-2 activity *in vivo* could be of clinical use. Our hypothesis is also compatible with recent results of Carnaud *et al.* (39) showing that the injection of semiallogeneic cells into new-born mice is followed by a nonspecific loss of 50% of precursors of IL-2-producing cells, accompanied by a larger specific decrease of 90% in the frequency of precursors of IL-2-producing cells responding to the injected alloantigens.

Since Ehrlich and Morgenroth (40) postulated "that the organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements," the mechanism of distinguishing "self" from "nonself" has been one of the most puzzling mysteries in biology. The present data, the negligible production of IL-2 in newborn mice (10) and the interference of exogenous IL-2 with the induction of neonatal tolerance (11) indicate indirectly that IL-2 could play an important role in this mechanism as well as in ontogenetically established nonreactivity to "self" antigens.

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