280

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Production of a Standardized Anti-Lymphocyte Globulin

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The production of a standardized human anti-lymphocyte globulin (ALG) has been hampered by the unavailability of a standard antigen for immunization, and by the finding that most immunization schedules produce variable results in different animals of a given species. In the present study it was found that horses immunized with cultured human lymphoblasts in a schedule of "maximum stimulation", all developed a very similar antibody response.

Methods

Preparation of ALG. Five horses were immunized with cultured human lymphoblasts. At the first immunization $65-140 \times 10^9$ cells were injected subcutaneously in *Freund*'s complete adjuvant (FCA) and at the same time $20-50 \times 10^9$ cells were given intravenously. Subsequently, 2 to 5 intravenous booster doses of $25-100 \times 10^9$ cells were given with 10–14 day intervals. In horse 5, three grams hydrocortisone was given with each of the booster injections. Five to 50% of the injected cells were viable.

Ten to 14 days after the last injection, the horses were bled to death. The horse serum was absorbed with human red cells, platelets and plasma, following which the globulin was precipitated with ammonium sulphate[1].

Testing of ALG. Lymphocytotoxic, leukoagglutinin, thromboagglutinin, and hemagglutinin titers were performed with standard techniques[2]. Immunosuppressive effect *in vivo* was studied in the rhesus monkey skingraft model at the National Institute of Health.

Pyrogen testing was carried out by injecting 4 cc ALG intravenously into rabbits; endotoxin was assayed for according to *Fritz* and *Nordenfelt*[3]. Antigenicity of the product was

studied by following precipitin titers (*Ouchterlony* gel), in rabbits injected twice with 3 cc ALG.

Results

Characteristics of the ALG. The 5 horses undergoing immunization developed very similar antibody responses (Fig. 1). One horse died immediately after the first booster injection

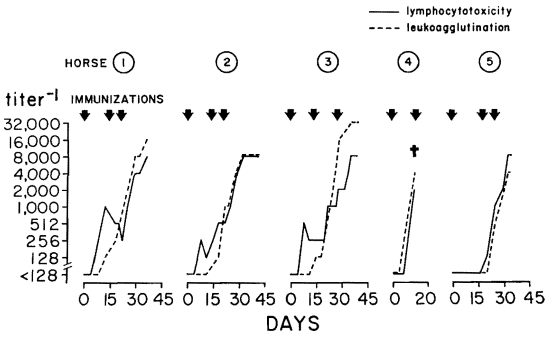

Fig. 1
Anti-white cell titers in 5 horses undergoing 3-pulse immunization with human cultured lymphoblasts.

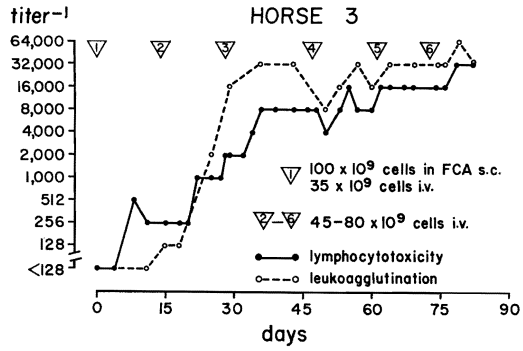

Fig. 2
Anti-white cell titers in a horse undergoing 6-pulse immunization.

probably of anaphylaxis. After 3 injections, the other 4 horses all had developed cytotoxic titers of 1 : 8000. At the same time leukoagglutinin titers were at least 1 : 8000 in all the animals. Horse 3 was given three additional boosters at the end of which both anti-white cell titers had risen to 1 : 32,000 (Fig. 2). Horse 5 is presently undergoing the same kind of extended immunization with the titers rising in a similar fashion.

Table 1:
In vitro characteristics of ALG prepared from the serum of 3 horses undergoing 3 or 6 pulse immunization with cultured human lymphoblasts.

	Horse 1 3-pulse	Horse 2 3-pulse	Horse 3 6-pulse
Lymphocytotoxic titer	4,000	4,000	32,000
Leukoagglutinin titer	8,000	8,000	16,000
Thromboagglutinin titer	16	32	64
Hemagglutinin titer	1,000	8,000	512
Protein content (g%)	8.2	6.4	7.6

The *in vitro* titers of the globulins prepared from the serum of horses 1 – 3 are depicted in Table 1. The results of the tests for pyrogenicity, endotoxin effect and antigenicity are summarized in Table 2.

Table 2:
Toxicity of 2 batches of human ALG in the rabbit.

Test	Horse 1 3-pulse	Horse 3 6-pulse
Pyrogenic response	0.6° C	1.2° C
Endotoxin effect	–	+
Precipitin antibody titer	1 : 2	1 : 4

When given a total dose of 1 g/kg, the ALG prepared from serum of horse 3 prolonged skin graft survival in a monkey to 22–24 days (controls less than 12 days).

Clinical trial. The ALG has been given to 26 renal transplant recipients. Thirteen patients had living related, and 13 had cadaveric donors, respectively. Approximately 4 mg/kg/day was given intramuscularly for 8 days to 3 months; the dose was daily for 2 weeks and then less frequently.

When given alone prior to the combined immunosuppressive regimen with prednisone and azathioprine or cyclophosphamide, the ALG decreased the peripheral lymphocyte count in 4 patients from an average of 2100 to 1300 lymphocytes per mm^3. In each of the same patients at least one positive delayed hypersensitivity skin reaction for mumps, candida, streptokinase or vaccinia was decreased; totally, 5 out of 7 such reactions were diminished. Pain and induration at the site of injection and moderate fever were common; significant thrombocytopenia and generalized skin reactions were never seen. Two patients that received ALG prepared from the serum of horse 3, developed an anaphylactic reaction with therapy resistent circulatory collapse and pulmonary edema leading to death. One of the patients had received ALG without the protection of simultaneously administered prednisone and azathioprine for the first 2 days. The other had the administration of ALG discontinued and then reinstituted 6 days later in connection with 2 consecutive transplantations. The anaphylactic reactions occurred after 2 and 7 weeks of ALG administration, respectively.

Sera from the 2 patients were studied simultaneously with sera from several other patients receiving ALG of the same batch and from other patients receiving other batches of ALG. In assaying precipitin and passive hemagglutinin titers against horse protein, and passive cutaneous anaphylaxis in the guinea pig, no distinguishing features were found for the 2 patients.

With 1 to 9 months of follow-up there has been no patient loss further to the 2 cases undergoing anaphylactic reaction. One graft has been removed following an early urological complication.

Discussion

The cultured human lymphoblast offers the purest and best standardized antigen for the production of ALG[4] and has the additional advantage that it can be made available in large quantities. When given in an immunization schedule characterized by the use of FCA with the initial subcutaneous injection, intravenous booster doses, and the use of large number of cells with each injection, a very similar antibody response was obtained in 5 horses. The resulting ALG had high anti-white cell titers *in vitro*, was immunosuppressive in the monkey skin graft model and induced peripheral lymphopenia and abolished delayed hypersensitivity skin reactions in patients. Side effects were moderate except in the 2 cases where anaphylactic shock led to the death of the patient.

Several studies were performed trying to elucidate the cause of these lethal complications which were the first to occur at our institution where ALG has been used for almost 6 years in more than 300 patients. The pertinent batch was pyrogenic and when retrospectively tested for endotoxin the material gave a positive result. In addition, it was more immunogenic in the rabbit than another batch tested. These findings might explain the untoward effects and underlines the importance of stringent *in vitro* toxicity control of ALG.

Based on the experience with 5 horses we propose an immunization schedule as outlined in Table 3. The hydrocortisone is administered to prevent anaphylactic reactions in the horse. With this program the number of cells required for the immunization of one horse is 250–500

Table 3: Proposed schedule for raising high titer ALG with cultured human lymphoblasts.

Day 0	100×10^9 cells in FCA s.c. and 25×10^9 cells i.v.
Days 14, 24, 34, 44, 54	$25-75 \times 10^9$ cells i.v. with 3 mg hydrocortisone
Days 60–70	Bleeding of horse

$\times 10^9$ and the procedure is completed in 2 months. The expected yield of ammonium sulphate precipitated ALG will be approximately 450 g from one horse. With the doses presently employed, that is a total of 10–20 mg globulin per patient, one horse can supply ALG for the treatment of 45 to 22 patients.

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