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CORRELATION OF THE IN VIVO AND IN VITRO ACTIVITIES OF ANTITHYMOCYTE SERA (ATS) WITH THE IMMUNIZING ANTIGEN DOSE[1]

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SUMMARY

Rabbits were immunized with 1, 3, 10, 30, 100, 300, or 1,000 $\times 10^6$ murine thymocytes per kg according to the method of Levey and Medawar. Thus, 33 individual and 6 pooled anti-mouse antithymocyte serum (ATS) preparations were obtained and tested for in vivo immunosuppressive (graft-protective) as well as for in vitro thymocytotoxic activity. It was found that: (1) at least 3×10^6 thymocytes/kg were necessary for inducing ATS of appreciable immunosuppressive activity; (2) rabbits immunized with 30×10^6 thymocytes/kg supplied sera of the most potent immunosuppressive activity; (3) the increase of the immunizing antigen dose over 30×10^6 thymocytes/kg resulted in ATS preparations of decreased immunosuppressive activity; (4) the graft-protective activity of an ATS pool corresponded to the average of the activities of the individual ATS preparations from which the pool had been mixed, i.e., the process of pooling itself did not modify the immunosuppressive activity; and (5) there was a good correlation ($r = 0.72$, $P < 0.001$) between the in vivo immunosuppressive (graft-protective) activity and the in vitro thymocytotoxic titre of ATS preparations. The theoretical and practical significance of these results is discussed.

The method of immunization described by Levey and Medawar (*11*) is widely acknowledged as a simple and reliable procedure to prepare antilymphocyte serum (ALS). By using this immunization method, it was observed that thymocytes are highly superior either to lymph node or to spleen cells in preparing rabbit anti-mouse ALS of powerful immunosuppressive (graft-protective) activity (*15*). Furthermore, it has been reported that antithymocyte sera (prepared according to the Levey-Medawar method) are preferable, too, because they produce minimal toxic inflammatory reactions at the site of injection (*18*). In addition, we found that, by using thymocytes (in contrast to lymph node or spleen cells) as targets in determining the cytotoxic titre of rabbit anti-mouse antispleen, antilymph node, and antithymus sera, there is a very good correlation between the graft-protective activity and thymocytotoxic titre of ALS preparations (*17*).

Thus, having selected a proper immunization method and immunizing antigen, our attention has been focused on the application of the appropriate amount of antigen to produce potent antithymocyte serum (ATS). Another purpose of this study was to determine the correlation of the in vivo graft-protective activity and the in vitro cytotoxic titre of ATS preparations produced by a wide range of antigen doses.

MATERIALS AND METHODS

Animals. New Zealand White rabbits (2.5 to 4.0 kg) purchased from a local breeder and isogeneic CBA ($H\text{-}2^k$) and A ($H\text{-}2^a$) mice bred in our laboratory by strict brother and sister matings were used. All of the animals received commercial rabbit or mouse food pellets, respectively, and water ad libitum.

Preparation of ATS. Rabbit anti-mouse ATS preparations were raised according to the two-pulse i.v. method of Levey and Medawar (*11*). In short, the rabbits were given 1, 3, 10, 30, 100, 300, or $1,000 \times 10^6$ murine thymocytes/kg on two occasions 14 days apart, and were exsanguinated 7 days after the second immunization. The thymocytes were obtained from 3- to 6-week-old female CBA mice. (For further details on the preparation of ATS see Reference (*15*).)

Skin grafting assay. Young adult male CBA mice (20 to 25 g) served as recipients and adult A mice as donors throughout the experiments. The technique of skin allografting and estimation of graft survival was based on the method of Billingham and Medawar (*1*) and has been described previously (*15*).

Assay of in vivo immunosuppressive activity of ATS. The in vivo immunosuppressive activity of ATS preparations was determined by measuring their ability to prolong the survival of A skin allografts on CBA mice. Recipients were given 2×0.5 ml of ATS s.c. on days 2 and 5, the day of grafting being day 0. One group of mice was given injections of 2×0.5 ml of pooled normal rabbit serum. Statistical analysis (Student's t test) of graft survival times served as a basis for comparison of the immunosuppressive potency of sera.

Assay of in vitro cytotoxic activity of ATS. Thymuses were obtained from 3-week-old female CBA mice. Single-cell thymocyte suspensions were prepared in Parker 199 tissue culture medium containing 10% calf serum (pH 7.3). Thymocytotoxic titres of ATS preparations were determined by the trypan blue dye exclusion test as described earlier (*17*).

RESULTS

The in vivo immunosuppressive activity of 33 individual ATS preparations as measured by their effect on the survival of A skin allografts on CBA mice was determined. Furthermore, 6 pools of ATS composed by mixing equal volumes of individual ATS of rabbits given the same amounts of antigen were also tested for graft-protective activity. Thus (including one pool of normal rabbit serum), 40 serum preparations were tested for in vivo activity. The experiments were terminated at day 120. The in vitro thymocytotoxic activity of the sera was also determined.

Correlation of the immunizing antigen dose with the immunosuppressive activity of ATS. Table 1 shows that all ATS preparations raised with 1,000, 300, 100, 30, or 10×10^6 thymocytes/kg possessed "strong" immunosuppressive activity. (According to our arbitrary scale, any ALS preparation has been considered to be of strong activity if its administration resulted in more than 20 days mean allograft survival under conditions described in detail in Materials and Methods.) In contrast, only one of four ATS preparations raised with 3×10^6

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TABLE 1. Graft-protective activity and thymocytotoxic titre of "individual" and "pooled" rabbit anti-mouse ATS preparations

No. of experiment	ATS preparation (×10⁶ thymocytes/kg)	Mean graft survival in days (±SE)	Range of graft survival	No. of grafted mice	Thymocytotoxic titre
1	1000/A[a]	52.6 (7.44)	30->120 (2)[b]	16	1280
2	1000/B	48.7 (4.13)	34-120 (1)	20	1280
3	1000/C	44.0 (5.70)	25-120 (1)	17	640
4	1000/D	31.0 (2.06)	18-64	20	320
5	1000 pool	45.4 (2.96)	32-71	16	1280
6	300/A	54.2 (4.88)	31-94	19	2560
7	300/B	51.1 (6.70)	17->120 (2)	18	640
8	300/C	50.7 (6.30)	25->120 (1)	18	1280
9	300/D	48.1 (4.38)	32-110	21	2560
10	300/E	43.6 (4.99)	22->120 (1)	20	2560
11	300/F	43.4 (4.12)	30->120 (1)	21	1280
12	300/G	36.4 (2.52)	24-71	20	640
13	300/H	30.5 (1.60)	19-49	21	1280
14	300/I	20.3 (0.55)	16-24	20	640
15	300 pool	43.2 (3.38)	20->120 (5)	57	1280
16	100/A	46.2 (5.10)	27->120 (1)	20	640
17	100/B	38.6 (3.21)	20-73	21	640
18	100/C	26.6 (3.65)	14-89	20	320
19	100/D	23.4 (2.41)	13-61	21	320
20	30/A	69.6 (7.13)	22->120 (4)	20	2560
21	30/B	61.1 (8.46)	22->120 (5)	20	640
22	30/C	55.0 (7.31)	27->120 (4)	21	640
23	30/D	36.3 (6.39)	20->120 (1)	16	320
24	30 pool	63.9 (8.38)	29->120 (3)	16	1280
25	10/A	55.8 (8.04)	25->120 (2)	17	1280
26	10/B	54.0 (6.20)	28->120 (1)	16	2560
27	10/C	39.7 (2.44)	32-57	12	1280
28	10/D	34.0 (6.82)	21->120 (1)	14	1280
29	10 pool	53.4 (6.62)	14->120 (5)	31	1280
30	3/A	22.2 (1.14)	15-34	18	320
31	3/B	17.8 (0.59)	13-23	21	640
32	3/C	15.6 (0.49)	11-19	19	320
33	3/D	15.5 (0.42)	13-19	15	160
34	3 pool	17.6 (0.71)	9-23	17	320
35	1/A	17.6 (1.40)	10-32	17	80
36	1/B	14.0 (0.36)	9-18	37	160
37	1/C	13.7 (0.46)	9-18	20	10
38	1/D	13.6 (0.53)	9-17	16	10
39	1 pool	12.6 (0.52)	9-16	17	40
40	Normal serum pool	13.7 (0.49)	10-18	20	10

[a] A, B, C, etc. denotes individual ATS preparations.
[b] Number of grafts still living at day 120 in parentheses.

thymocytes/kg possessed strong, while three of them exhibited "weak" activity. (In our terminology, weak activity means that an ALS preparation results in less than 20 days mean graft survival, but at the same time, its application produces statistically significant prolongation of allograft survival compared with the mean graft survival observed on normal rabbit serum-treated control animals.) ATS preparations raised against 1×10^6 thymocytes/kg did not show graft-protective activity, with the exception of one serum having weak activity. Thus, using less than 10×10^6 thymocytes/kg as antigen dose, there was a sharp decline in the immunosuppressive activity of the ATS preparations. Further analysis of the data shows that the increase of the number of thymocytes above 30×10^6/kg did not result in further increase of the in vivo activity of ATS preparations. On the contrary, pooled sera obtained after immunization either with 300×10^6 or with $1{,}000 \times 10^6$ thymocytes/kg exhibited weaker activity than the ATS pool raised with 30×10^6 thymocytes /kg. (The difference is statistically significant, see Table 2.)

Effect of pooling on the immunosuppressive activity of ATS preparations. Table 1 shows that there was a rather wide variation in the immunosuppressive activity of the ATS preparations raised in individual rabbits, even if the rabbits were given identical amounts of antigen. Therefore, it was determined whether the process of pooling of sera raised with the same amount of antigen would result in an average immunosuppressive activity of the pool as calculated from the graft-protective effect of its components, or whether the process of pooling itself would shift the immunosuppressive activity in any direction, as observed by Lamoureux et al. (*10*), by mixing ALS preparations raised with lymphoid cells of different organs.

Table 2 comprises the "real" mean graft survival times found after the application of pooled sera compared with the "calculated" graft survival times (i.e., the mean graft survival times determined by summing up and averaging the survival times of all of the corresponding individual serum). Statistical analysis showed that the calculated mean graft survival did not differ significantly from the real mean graft survival of the ATS pools.

TABLE 2. Comparison of the graft-protective activity of grouped individual versus pooled ATS preparations

No. of experiment	ATS preparation ($\times 10^6$ thymocytes/kg)	Mean graft survival in days (±SE)	Range of graft survival	No. of grafted mice	Group comparison	P
1-4	1000/A + B + C + D[a]	43.6 (2.59)	18->120 (4)[b]	73	1-4 versus 5	NS[c]
5	1000 pool	45.4 (2.96)	32-71	16	5 versus 24	<0.01
6-14	300/A + B + C + D + E + F + G + H + I	41.7 (1.61)	16->120 (5)	178	6-14 versus 15	NS
15	300 pool	43.2 (3.38)	20->120 (5)	56	15 versus 24	<0.01
20-23	30/A + B + C + D	56.5 (3.90)	20->120 (14)	77	20-23 versus 24	NS
24	30 pool	63.9 (8.38)	29->120 (3)	16		
25-28	10/A + B + C + D	46.8 (3.47)	21->120 (4)	59	25-28 versus 29	NS
29	10 pool	53.4 (6.62)	14->120 (5)	31		
30-33	3/A + B + C + D	17.8 (0.47)	11-34	73	30-33 versus 34	NS
34	3 pool	17.6 (0.71)	9-23	17		
35-38	1/A + B + C + D	14.5 (0.36)	9-32	90	35-38 versus 39	<0.05
39	1 pool	12.6 (0.52)	9-16	17		

[a] A,B,C, etc. denotes individual ATS preparations.
[b] Number of grafts still living at day 120 in parentheses.
[c] NS, not significant.

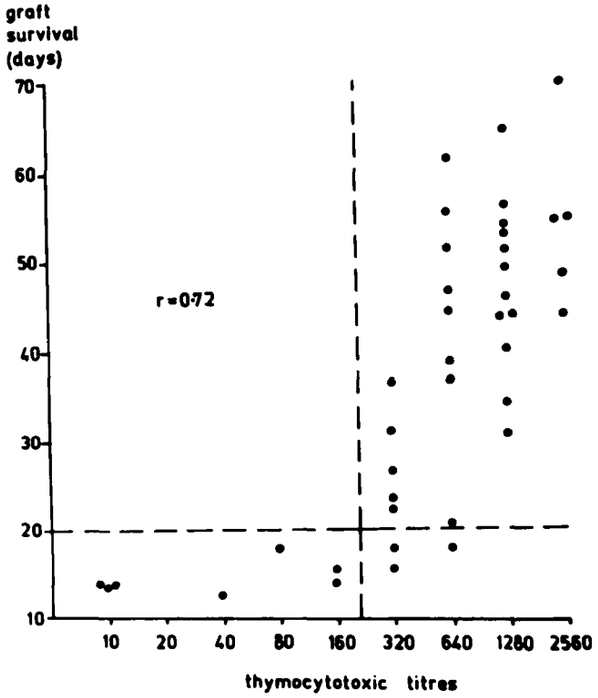

FIGURE 1. Correlation of the graft-protective and thymocytotoxic potency of ATS preparations. Each dot represents the mean values of a single ATS preparation.

(The only exception was the 1×10^6 thymocytes/kg ATS pool, where there was a statistically significant ($P < 0.05$) but biologically insignificant (12.6 versus 14.5 days) difference between the real and the calculated means.)

Correlation of the in vivo immunosuppressive activity and the in vitro thymocytotoxic titre of ATS preparations. The thymocytotoxic titres of 33 individual and 6 pooled ATS preparations as well as of 1 normal rabbit serum pool were determined and correlated with their graft-protective activity. The thymocytotoxic titres were expressed as the highest dilution of serum preparations resulting in more than 30% stained cells. Thymocytotoxicity tests were performed at least on three different occasions for each serum preparation and the mean titres were calculated (see Table 1). (It should be noted that the thymocytotoxicity test, as performed by us (*17*), gave highly reproducible results. Determination of the cytotoxicity of a given serum preparation on different occasions revealed, in most cases, identical titres, or, if not, the differences in the titre values did not exceed ± 1 degree of serum dilution.)

Figure 1 shows that there was a fairly good correlation ($r = 0.72$, $P < 0.001$) between the thymocytotoxic and immunosuppressive activity of the sera. Furthermore, one may see that: (1) none of the sera with less than 320 thymocytotoxic titre revealed strong immunosuppressive activity; (2) most of the sera with more than 160 thymocytotoxic titre had strong immunosuppressive activity (30 from 33 sera); and (3) all sera with more than 640 thymocytotoxic titre exhibited strong activity.

DISCUSSION

The importance of the determination of the optimal antigen dose needed for the production of ATS preparations revealing high immunosuppressive activity and minimal toxicity is self-evident both from theoretical and economical reasons. To our surprise, we found an almost complete lack of literary data in this respect. Thus, we were inspired to perform a detailed comparative study on the correlation of the immunizing antigen dose used for raising ATS preparations and of the immunosuppressive activity of the serum preparations obtained.

The simplicity and reliability of the immunizing method of Levey and Medawar (*11*) to produce ALS preparations with good immunosuppressive (graft-protective) activity is widely acknowledged. Another advantage of this technique is that the ALS preparations obtained are the least toxic in a clinical sense, while the same cannot be said about sera prepared with the use of adjuvants (*8, 9, 12*). Thymocytes proved to be especially suitable to prepare ALS of strong immunosuppressive activity and minimal toxicity in both our and others' hands (*5, 13, 15, 18*).

In the present studies, we used a wide range of different antigen doses for the immunization of rabbits. Data in Table 1 show that the injection of 30×10^6 thymocytes/kg seems to be the most advisable amount of antigen to prepare ATS with

optimal immunosuppressive activity. This amount is approximately 10 times less than the antigen dose generally used.

Our observations seem to be somewhat contradictory to the remark of Levey and Medawar (*11*) about the amount of antigen, "the more the better." The contradiction can be resolved if we consider that they injected 400 to 1,000 million cells/rabbit; the use of more and especially less cells for immunization is not mentioned. The same relates to the observation of Jooste et al. (*8*), who found that the strength of sera raised by a single i.v. injection " . . . were not much affected by the size of the immunizing dose over a wide range;" they used 10^8, 10^9, or 10^{10} thymocytes/rabbit. Here again the use of a lower number of cells is not mentioned. Gozzo et al. (*3*) raised ALS or ATS by an adjuvant method (immunizing cells were emulsified in Freund's complete adjuvant). Because of the differences in the experimental methods, their and our results are difficult to compare. There is, however, at least one point of agreement, namely, that a relatively low number of thymocytes was enough to produce potent ATS in rabbits in their and our experiments. However, the danger of producing irrelevant antibodies by the adjuvant method is seriously stressed by the cited authors and also by others (*3, 8, 9*).

An unexpected finding of our study was that by raising the immunizing cell dose over an "optimal" level ($=30 \times 10^6$ thymocytes/kg), a moderate but definite decline in the immunosuppressive potency of ATS preparations had been observed. Thus, a similarity seems to exist between the well known decay phenomenon (*2, 3, 6–8, 14*) and our results insofar as in both cases the decay of immunosuppressive activity is caused by some kind of "overshooting" by antigen excess; the difference is that while in the case of the "classical" decay phenomenon, the overdosage of antigen is brought about by multiple antigen injections and in our case it is the amount of antigen itself that matters in the induction of the decay. In our knowledge, this is the first description of such decline of potency related to the production of ALS.

Lamoureux et al. (*10*) reported that the immunosuppressive activity of ATS was potentiated when mixed with antispleen, antilymph node, or antibone marrow ALS. We observed (*16*) that absorption of ATS with a limited number of lymphoid cells increased the immunosuppressive activity, whereas absorption with a larger number of cells decreased it. Both observations might be explained by taking into account the extreme heterogeneity not only of the immunizing antigen but also of the resulting antibodies of different activities (*4*). Thus, it did not seem unreasonable to examine whether the process of pooling itself would modify the immunosuppressive activity of the ATS preparations. No such modifying effect could be revealed in our study. On the contrary, the immunosuppressive activity of any ATS pool could be well predicted by summing up and averaging the mean activity of the single, individual ATS preparations from which the pool had been mixed.

We described earlier (*17*) that there was an excellent correlation between the immunosuppressive potency and the thymocytotoxic titre of antispleen, antilymph node, and antithymus antilymphocyte sera. The source of the target cell in determining the cytotoxic titre of sera was crucial: the correlation was far worse if spleen or lymph node cells were used (instead of thymus cells) as targets. In the present experiments we could confirm and extend our previous observations: thymocytotoxic titres correlated very well with the immunosuppressive activity also in the case of ATS preparations raised with different amounts of antigen. However, the following restrictions based on our previous and present experience should be emphasized: (1) Thymocytotoxic antibodies should not be considered to be identical to immunosuppressive antibodies, a parallelism rather than identity of the two kinds of antibodies is supposed. Hence, the determination of the thymocytotoxic titre is extremely helpful in classifying the ALS preparations into "ineffective," weak, "good," or "very good" sera in regard to their immunosuppressive activity; however, one should not expect the precise prediction of the graft-protective effect of an ALS preparation on the basis of its titre. (2) The very close correlation observed by us does not necessarily apply to ALS preparations raised either in other species or by means of other immunization methods. These sera may have different in vivo or in vitro characteristics (*19*).

Finally, it should be stressed again that we obtained the best sera by immunizing rabbits with 30 million thymocytes/kg, i.e., approximately with one-tenth of the cell number applied generally. Bearing in mind the danger of extrapolation of our data to other systems, we point but to the potential importance of this observation not only in producing ALS preparations for experimental purposes, but particularly in producing antihuman ALS for clinical use, where one of the limiting factors is the shortage of the sufficient amount of antigen, be it derived either from fresh human thymocytes, thoracic duct lymphocytes, or, e.g., cultured human lymphoblasts.

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