The Effect of Isoantibodies *in Vivo* on Three Different Transplantable Neoplasms in Mice

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It is now well established that transplantable tumors in mice react characteristically to contact, *in vitro* and *in vivo*, with putatively cytotoxic isoantisera[1] (9, 18). Some tumors, specifically the leukoses,[1] are almost completely destroyed (3, 9–11); others, like the C3H sarcoma B.P.8, are affected to a lesser degree (8–10); while tumors, like Sarcoma I, appear almost completely insensitive[1] ([10]; Gorer and O'Gorman, unpublished data). Tumors in the second and third categories are of interest, since they may exhibit "immunological enhancement" after contact with antiserum (10, 14, 15). Gorer ([9], and unpublished data) has described the alternatives of either inhibition or enhancement of subcutaneous homografts of B.P.8 in BALB/c and strain A mice, following the exposure of the tumor cells *in vitro* to different concentrations of BALB/c anti-B.P.8 serum. Exposure to smaller quantities of antiserum produced enhancement, while inhibition followed exposure to larger quantities. The serum quantities ranged, in approximately doubling dilutions, from 0.025 to 0.25 ml. These observations prompted the present studies, which were designed to compare the behavior of homografts of tumors, falling into the three categories described above, when exposed to different dosages of antisera *in vivo*, and in different inbred "foreign" strains. (The present experiments have been alluded to in outline in previous publications [10, 14].)

MATERIALS AND METHODS

The test tumors have been described in detail previously. They are the C57 Black leukosis, E.L.4 (11), the C3H sarcoma B.P.8 (8, 10), and the strain A tumor, Sarcoma I (SaI) (10, 15). They are maintained in the ascites form by routine transfer in mice of the native strain. For injection into the experimental animals (and for routine transfer), the distended abdomens of the tumor-bearing mice were tapped with a sterile pipette, usually about 14 days after the animals had been inoculated. To prevent clotting, the ascitic fluid was expelled into a mixture (5:1) of sterile Ringer's solution and 3 per cent sodium citrate. Cell counts were made, and the cell suspension was further diluted as necessary to give the cell concentration desired for injection. In the experimental animals, the sarcoma cells (B.P.8 and SaI) were injected in a volume of 0.05 ml. into the right calf muscle. One has the choice of intraperitoneal, subcutaneous, or intramuscular inoculation. The first of these is not suitable for the present experiments, since the fate of the tumor cells cannot be followed without operative interference. The subcutaneous route is the most advantageous for morphologic studies and accuracy of measurement of graft size. However, enhancement of B.P.8 is somewhat capricious at this site, and the intramuscular route was therefore adopted with this tumor (and with SaI, for comparable determinations). The leukemia E.L.4 was injected subcutaneously, in accordance with previous experience (11).

Growth of the grafts was followed by periodic measurements of the graft site with calipers. The index of size, as shown in the accompanying tables, is the sum, in mm., of two diametrical measurements taken at right angles to each other. The initial increases in size of the inoculated muscle, though obvious as such, were too small to measure with calipers, and they were therefore classified as "±," "+," or "++." In tabulating the data, these categories have been given the arbitrary values of 2, 6, and 10, respectively. Observations were made approximately twice weekly for the first 3 or 4 weeks after tumor inoculation, and

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[1] H. J. Winn, Immune Mechanisms in Tumor Homotransplantation I. The Role of Complement and Serum Antibody (to be published).

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weekly thereafter until death of the animals that bore progressive tumor growths. Animals that had remained without any obvious sign of an enlarged calf muscle region for a consecutive period of at least 2 months were classified as "negative."

The antisera were hyperimmune isoantisera to the tumors; the method of their production has been described previously (11). The source of each serum will be given below in the description of the particular experiment. The sera were injected intraperitoneally, in the required dosage, from ½ to 1 hour before inoculation of the tumor homograft.

RESULTS

Tumor B.P.8. in BALB/c hosts.—Two experiments are reported, both similar in design, but with different batches of isoantisera and slightly different dosage ranges. Both antisera were BALB/c anti-B.P.8. (In terms of the H-2 antigenic system [see 4], the antisera would be H-2d anti-H-2k; i.e., they would be directed against the known H-2 antigens, E and K.) In Experiment 1, the tumor cells were inoculated in the calf muscle in the amount of 2.8 million cells/0.05 ml/mouse. In Experiment 2, the quantity of cells per mouse was 2.5 million. The ranges of antiserum dosages injected, in ml/mouse, were: Experiment 1: 0.2, 0.1, 0.05, and 0.025; Experiment 2: 0.4, 0.2, and 0.05.

The results for Experiment 1 are tabulated in Table 1. There were three males and three females in each serum dosage group and in the control groups; the data are presented as the mean tumor size for each sex. It is evident that the passively transferred antiserum enhanced the growth of the tumor grafts in all the groups. However, in terms of tumor size on any given day, the most rapid growth of the grafts was in the groups receiving the lower serum doses; this is particularly evident with the dose of 0.05 ml. and is most clearly shown in the females. Furthermore, in the 0.2-ml. group, there was an apparently complete regression for about 10 days after the initial positive record, in the one positive female and one of the two positive males. In the other experimental groups, the positive animals showed continued progressive growth throughout. There actually may have been some inhibition of growth at the highest dose level (0.2 ml.) if one compares the mean values for the injected and control males on day 8. (This is shown more clearly in Experiment 2, to be described below.) A suggestion of inhibition also appears in a comparison of the individual animals on day 8; there was definite enlargement of the injected calf in all six control mice (three males and three females), while one each of the three males and three females in the 0.2-ml. group was recorded as negative. There are several other observations of interest which are not brought out in the table. In most of the mice with progressive tumor growths necrosis of the tumors set in as they became very large. This first became evident by the 29th day in the mice receiving 0.025–0.1 ml. of serum, and by 42 days in two of the three mice dying with tumors in the 0.2-ml. group. Necrosis of the leg and foot occurred in many of the mice, and in one male in the 0.05-ml. group the leg with its tumor withered to the point where it dropped off completely. The mouse remained alive without any further sign of a tumor for 1 month, at which time it was killed. This "autoamputation" also occurred in one male in the 0.025-ml. group. Two weeks after this, the mouse received 27.2 million B.P.8 cells subcutaneously, and it died with a progressively growing tumor 2 months later. Though neither of these mice appears in the "No. dead" column of Table 1, they should properly be considered to have had enhanced tumor grafts.

TABLE 1

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF GRAFTS OF THE C3H TUMOR, SARCOMA B.P.8, IN BALB/C HOSTS

The antiserum was BALB/c anti-B.P.8. This represents the results of Experiment 1 in the text.

SERUM DOSE	AVERAGE GRAFT SIZE ON INDICATED DAY AFTER GRAFTING*								No. DEAD/ TOTAL
(ml/mouse)	8	11	17	21	29	36	42	48	
Male hosts:									
0.2	7	3	7	9	17	21	28	v.l.	2/3
0.1	19	25	31	35	40	v.l.			3/3
0.05	21	28	35	39	v.l.				2/3
0.025	20	28	32	33	37	v.l.			2/3
Control	13	0	—	—	—	—	—	—	0/3
Female hosts:									
0.2	4	0	0	5	8	11	12	v.l.	1/3
0.1	12	15	19	15	18	v.l.			2/3
0.05	18	21	28	27	36	36			3/3
0.025	14	16	21	21	24	v.l.			2/3
Control	4	0	—	—	—	—	—	—	0/3

* The figures for graft size are the sum of two diametrical measurements in mm., taken at right angles to each other; v.l. = very large; no caliper measurements were made at this stage. — = all mice were negative.

Experiment 2.—There were five males and five females per group. Since there appeared to be an indication of inhibition on day 8 in Exp. 1 (in the males), an earlier first observation was now made in the hope of getting clearer evidence of this. The data are given in Table 2. There clearly was inhibition at 5 days in the groups of females receiving

0.4 or 0.2 ml. of serum, as compared with the controls, and there may be some evidence of this in the comparable groups of males. In individual comparisons of the mice in the 0.4-ml. group and the controls at this time, all of five males and four of five females in the controls had an enlarged inoculum site, while four of five males and three of five females were "positive" in the serum-treated group. As in Exp. 1, the most rapid increase in tumor size (enhancement) was in the mice receiving the lower serum dosages. This is particularly evident in the mice receiving 0.05 ml., and the contrast is most striking in the females.

TABLE 2

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF GRAFTS OF TUMOR B.P.8 IN BALB/C HOSTS

The antiserum was BALB/c anti-B.P.8. This represents the results of Experiment 2 in the text.

Serum dose	Average graft size on indicated day after grafting*								No. dead/ Total
(ml/mouse)	5	8	14	16	20	23	27	33	
Male hosts:									
0.4	4	10	12	11	19	21	26	30	4/5
0.2	4	6	13	14	20	27	29		5/5
0.05	7	18	25	25	29	31	32	v.l.	5/5
Control	5	1	—	—	—	—	—	—	
Female hosts:									
0.4	2	1	4	4	4	5	5	6	1/5
0.2	1	5	6	7	11	13	14	19	3/5
0.05	7	18	27	28	30	31	36	v.l.	5/5
Control	5	0	—	—	—	—	—	—	

* See footnote, Table 1.

Tumor B.P.8 in A/Go hosts (Experiment 3).—The isoantiserum was A anti-B.P.8 (H-2a anti-H-2k). The serum had been demonstrated to have cytotoxic activity against the tumor *in vitro* by Dr. P. O'Gorman (unpublished data). Two serum dosages were used, 0.2 and 0.05 ml., respectively. The tumor cell dosage was 2.3 million cells per mouse. There were five males and five females per experimental group and four males and five females in the controls. The data for average tumor size are given in Table 3. In contrast to the results in BALB/c hosts, all the growths eventually regressed. However, the growths reached a much larger size, and persisted longer, in the mice receiving 0.05 ml. of serum. Furthermore, in comparing the group receiving 0.2 ml. of serum and the uninjected controls, there was definite evidence of *inhibition* at the larger serum dosage. The delayed appearance of a growth in the males of the 0.2-ml. group was due to one animal. There was no evidence of growth in the other four males. There was also a very slight evidence of growth (++) in one of the females in this group at 7 and 10 days after tumor inoculation. All of four males and five females in the control group had transient swellings of the inoculum site up to day 10, but were normal by day 14.

TABLE 3

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF TUMOR B.P.8 IN A/GO HOSTS

The antiserum was A anti-B.P.8. This represents the results of Experiment 3 in the text.

Serum dose	Average graft size on indicated day after grafting*						
(ml/mouse)	7	10	14	17	21	28	35
Male hosts:							
0.2	0	0	0	2	4	4	0
0.05	9	15	7	3	1	0	0
Control	8	7	0	0	0	0	0
Female hosts:							
0.2	0	0	0	0	0	0	0
0.05	5	8	3	0	0	0	0
Control	4	7	0	0	0	0	0

* See footnote, Table 1.

TABLE 4

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF TUMOR B.P.8 IN C57BL/GO HOSTS

The antiserum was C57BL/Go anti-B.P.8. This represents the results of Experiment 4 in the text.

Serum dose	Average graft size on indicated day after grafting*							
(ml/mouse)	5	7	10	14	19	27	33	54
Male hosts:								
0.2	8	20	24	29	v.l.	4	0	0
0.05	6	21	28	30	22	6	2	0
Controls	9	19	9	0	0	0	0	0
Female hosts:								
0.2	2	11	20	28	9	0	0	0
0.05	8	11	23	21	0	0	0	0
Controls	9	8	1	0	0	0	0	0

* See footnote, Table 1.

Tumor B.P.8 in C57BL/Go hosts.—Two experiments were done with this combination, with the use of two different isoantisera. In Experiment 4, the antiserum was C57BL/Go anti-B.P.8 (H-2b anti-H-2k). The antiserum was administered in one of two doses, either 0.2 or 0.05 ml/mouse; the inoculum dose was 2.5×10^6 cells. There were five males and five females per group. The data for the average tumor sizes are given in Table 4. Evident-

ly there was a transient but marked enhancement at both dosages of antiserum. There was a definite indication of inhibition on day 5 in the females given 0.2 ml. of serum. Of interest is the very large size attained by the enhanced growths before they regressed. In fact, on the basis of our experience with B.P.8 in BALB/c hosts (Exps. 1 and 2), we would have confidently predicted on day 14 that the tumors would continue growing progressively until the death of the hosts.

In Experiment 5, the antiserum used was A anti-B.P.8 (H-2a anti-H-2k). (This was a mixture of two lots of sera, one of which had been used for Experiment 3, described above.) The serum was injected at the one dosage of 0.2 ml/mouse, and the tumor dosage was 2.1×10^6 cells. There were five males and five females per group. The data are given in Table 5. There was no enhancement,

TABLE 5

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF TUMOR B.P.8 IN C57BL/GO HOSTS

The antiserum was A anti-B.P.8. This represents the results of Experiment 5 in the text.

SERUM DOSE	AVERAGE GRAFT SIZE ON INDICATED DAY AFTER GRAFTING*				
(ml/mouse)	7	9	12	15	42
Male hosts:					
0.2	17	9	3	0	0
Control	16	3	0	0	0
Female hosts:					
0.2	<1	0	0	0	0
Control	8	0	0	0	0

* See footnote, Table 1.

and there was evidence of inhibition in the serum-injected females, when compared with the controls.

E.L.4. in strain A/Go hosts (Experiment 6).—The effect of an A anti-E.L.4 serum (H-2a anti-H-2b) was tested on the C57 Black leukosis, E.L.4, in strain A/Go hosts. The serum was administered in the respective doses, per mouse, of 0.1, 0.05, and 0.01 ml. The dosage of tumor cells was 4×10^6 cells per mouse, inoculated subcutaneously. There were five males and five females per group. The average tumor sizes, with time, are shown in Table 6. Obviously, there was no enhancement of the tumors. On the contrary, there was clear evidence of inhibition at the two higher serum dose levels, and possibly also at the lowest serum dosage in the females.

Sarcoma I (SaI) in BALB/c hosts.—Two experiments are reported. BALB/c anti-SaI serum was used in both experiments, though the sera were not of exactly the same lots. In Experiment 7, a mixture of three lots of sera was used; one of these three was also used for Experiment 8. There were five males and five females per group.

Experiment 7.—The tumor was injected into the right calf muscle in a dosage of approximately 2.1×10^6 cells. This count is certainly not accurate, since the cells tended to clump. Enhancement occurred at all three dose levels of antiserum (0.2, 0.05, and 0.01 ml.), with no apparent difference in growth rates (Table 7). However, there was a dif-

TABLE 6

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF GRAFTS OF THE C57 BLACK LEUKOSIS, E.L.4, IN A/GO HOSTS

The antiserum was A anti-E.L.4. This represents the results of Experiment 6 in the text.

SERUM DOSE	AVERAGE GRAFT SIZE ON DAY*		
(ml/mouse)	6	9	13
Male hosts:			
0.1	0	0	0
0.05	1†	2†	0
0.01	26	15	0
Control	29	7	0
Female hosts:			
0.1	0	0	0
0.05	0	0	0
0.01	9	14	0
Control	24	12	0

* See footnote, Table 1.

† Represents transient growth in only one of five males in the group.

ference in survival times for the two higher serum dosage levels, the times for both sexes being markedly and significantly shorter for the 0.05-ml. group. The spread in survival times was also much smaller in this group, in comparison with the other two dosage groups, as is shown by the standard errors of the means

In *Experiment 8*, which was started 2 months after the start of Exp. 7, 1.5×10^6 cells were injected into the right calf muscle. The serum was administered in two dose levels, 0.2 ml. and 0.05 ml. Again, there was enhancement at both dose levels, with no apparent differences between the two groups. In contrast to Experiment 7, there was no significant difference in survival times. Also, three tumors grew progressively in the controls, in two males and a female. In the two males, one mouse showed consistent progressive growth of the tumor, and the animal died 47 days after tumor

inoculation; the second male showed a slight decrease in size of the growth during the first 3 weeks. The foot, below the level of tumor growth, withered and subsequently fell off. The tumor became large and necrotic, and the animal died 54 days after tumor inoculation. In the female, there was an apparent complete regression of the tumor by the 2d week, after an initial period of growth. Growth was again evident a week later, and the animal died 40 days after inoculation of the tumor.

TABLE 7

EFFECT OF ANTISERUM DOSAGE ON THE GROWTH OF GRAFTS OF SARCOMA I (STRAIN A TUMOR) IN BALB/C HOSTS*

The antiserum was BALB/c anti-SaI. This represents the results of Experiment 7 in the text.

SERUM DOSE	AVERAGE GRAFT SIZE ON DAY†				AV. SURVIVAL TIME ± STANDARD ERROR (DAYS)
(ml/mouse)	7	10	14	18	
Male hosts:					
0.2	23	28	45	v.l.	37±2.99
0.05	21	25	v.l.	v.l.	24±0.57
0.01	24	28	v.l.	v.l.	36±2.92
Controls	21	19	2	0	
Female hosts:					
0.2	23	27	v.l.	v.l.	56±4.47
0.05	26	29	v.l.	v.l.	29±0.45
0.01	23	27	v.l.	v.l.	31±1.37
Controls	19	14	1	0	

* All the experimental mice (five males and five females per serum dosage group) died with progressively growing tumors.

† See footnote, Table 1.

DISCUSSION

These experiments demonstrate some of the elements which determine the fate of a tumor homograft subjected to specific antiserum *in vivo*. They may be characterized as: (*a*) the sensitivity of the tumor to putatively cytotoxic antisera, (*b*) the specificities of the antiserum, (*c*) the manner in which the host reacts to the "foreign" graft, (*d*) the antigenic relationships of host and graft.

The effect of the site of tumor inoculation, apparently one determinant in how the host reacts to the graft, has been mentioned above. We do not know why the intramuscular site should have been superior to the subcutaneous site for our purposes. It may be associated with a perhaps more rapid transport of antiserum to the intramuscular site (see 1), or with the demonstrated more rapid cellular reaction to a homograft at this site (10). The onset of the homograft reaction in the muscle is about 48 hours in advance of that to subcutaneous inocula.

The three tumors used demonstrate the range of reactions to an antiserum *in vivo* which has been previously pointed out by Gorer (9) and Snell (18). Characteristically, the leukosis E.L.4 was inhibited; the sarcoma B.P.8 was either inhibited or enhanced (dependent upon the serum dosage); Sarcoma I reacted only with enhancement. It is emphasized that these reactions were determined by the conditions of our experiments. The importance of this stricture is underlined by Gorer's report (6) of the accelerated growth of a leukemia which had been exposed to antiserum *in vitro*, provided the mouse received a large inoculum of the exposed cells. Conversely, inhibition resulted if the inoculum was small. Winn[1] and Mitchison and Dube (16) have observed neutralization of Sarcoma I after exposure *in vitro* to an appropriate isoantiserum.

The most striking aspect of our experiments is the induction of either inhibition or enhancement of the tumor B.P.8, dependent upon the dosage of antiserum injected into the host. In considering the bases for these opposite results, two alternative postulates can be proposed: (*a*) a single species of antibody is acting, the dual results being dependent upon the relative concentrations of tumor cells and antiserum (other things being equal); or (*b*) two different types of antibodies are involved, one being "cytotoxic" and the other "enhancing." Subsidiary assumptions are necessary with both postulates.

With the first postulate, the following scheme might be envisioned. The tumor cells are in part vulnerable to cytotoxic damage by the antiserum (as has been demonstrated for B.P.8 [10]). On contact with the higher concentrations of antiserum, the proportion of cells injured is so large that there is either complete inhibition of tumor growth or a marked delay in onset of progressive growth. At the lower antiserum concentration, sufficient cells survive to give enhanced growth, without any initial retardation. The second postulate—that of two different types of antibodies—necessitates the assumption that the cytotoxic antibody is considerably less effective at the lower concentrations of antiserum, while the "enhancing" antibody can be effective at very low concentrations. For both postulates, enhancement takes place with the cells that have escaped destruction by antibody. (The mechanism of enhancement requires consideration apart from the questions under immediate discussion.)

Which of the two postulates is correct cannot be

definitively determined from the present data, though we are inclined to the first, that of a single antibody (or species of antibody), the duality of response in the host being determined by the relative concentrations of tumor cells and antibody, by the relative cytotoxic sensitivity of the tumor, by the characteristics of the host's reactions, and by the antigenic relationships between the graft and the host. Evidence for this can be adduced from the experiments wherein B.P.8 was tested in A/Go (Exp. 3) and C57BL/Go hosts (Exp. 5). Though antiserum of the same specificity (A anti-B.P.8) was used for both experiments, both inhibition and enhancement were produced in the A/Go hosts, while only inhibition occurred in the C57BL/Go mice (females). The difference in results was apparently due to inherent differences in the host strains' reactions.

A comparison of Exp. 5 with Exp. 4, in which the C57BL/Go strain was also used as the host, indicates that the nature of the antibody is of some importance. In contrast with Exp. 5, the mice of Exp. 4 exhibited both enhancement (a marked delay in regression) of the B.P.8 grafts at the lower serum doses, and inhibition in the females at the highest serum dose. In Exp. 5, the antiserum used was H-2a anti-H-2k (A anti-B.P.8), while in Exp. 4 it was H-2b anti-H-2k (C57BL/Go anti-B.P.8). The former contains an antibody that has been shown to be anti-D^k (12) (it probably contains a mixture of antibodies), while the latter contains anti-K (see references in [2, 12]) (C57BL/Go mice should be able to produce anti-D^k, but so far have not been observed to do so). It is not obvious why the differences in type of antibody should be accountable for the variant results of Experiments 4 and 5, since anti-D^k does not appear to be any more cytotoxic *in vitro* than anti-K. It could be due to some peculiarity of the "D^k" antigen(s), or some subtle property of the antibody molecules.

What bearing do the present experiments have on the mechanism of "immunological enhancement"? It has been maintained by one of us (N.K.) that enhancement is due to some (as yet obscure) "physiological alteration" of the tumor, as a consequence of its contact with antibody, which now permits the tumor to survive in the face of the hostile responses of the host. Alternatively, it has been postulated (5, 17) that enhancement is due to the absence of the normal immune responses of the host, since the antigens, now "blocked" by antibody, cannot provide an adequate immune stimulus. The reasons for our believing that "blockage" in this sense cannot account for enhancement have been set forth in detail elsewhere (14, 15) and need not be repeated here. The present data do not critically discriminate between the alternative hypotheses, since as yet we can only speculate about the basis of the serum-dosage effect. In the case of tumor B.P.8, we have postulated that the relative numbers of surviving tumor cells, at each serum-dosage level, will determine whether inhibition or enhancement follows exposure to antiserum (but as we have stated above, this does not explain enhancement itself). However, differential cell mortalities can hardly be the explanation for the results with SaI (see Table 7), since exposure to antiserum does not appear to kill this tumor[1] (10; Gorer and O'Gorman, unpublished data). Yet there appears to be an accelerated growth of SaI exposed to lower serum doses in Exp. 7 (actually, a significant shortening of survival time). (It should be pointed out that SaI cells, despite the apparent absence of cytotoxic damage by antiserum, as determined by the cell staining technic, may undergo some fundamental metabolic changes which may be expressed in the subsequent growth rates of the tumor.) "Blockage" could hardly explain these results, since it would be expected that the larger dose of antiserum would be at least as effective in blocking as the smaller ones. However, though enhancement cannot be explained satisfactorily by simple "blockage," the possibility is not eliminated that the presence of excess antibody may have important effects in altering the cellular responses of the host. (As one of us has pointed out [P.A.G.] [9, 10], the successful "enhancement" of normal tissue grafts probably demands such effects.)

SUMMARY

Homografts of two sarcomas and one leukemia were made in mice receiving injections of isoantisera. With one sarcoma, there was inhibition of growth in mice receiving relatively large doses of antiserum, while enhancement took place at the lower serum doses. The dual response was demonstrable with the sarcoma in two inbred strains of mice, whereas only enhancement of the tumor took place in a third strain. With the second sarcoma, no inhibition was observed, but there appeared to be a more rapid rate of enhanced growth at the lower serum dosage. With the leukemia, only inhibition took place.

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