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SURFACE MARKERS ON THE T CELLS THAT REGULATE CYTOTOXIC T CELL RESPONSES

II. Distribution of Ly 6.1 and Ly 7.2: Selective Expression of Ly 7.2 on Helper T Cell Effectors[1]

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Helper T cells and suppressor T cells can be generated in vitro, yielding effector cells with radioresistant function that regulate a cytotoxic T cell response to alloantigen. The Ly 6.1 and Ly 7.2 antigen phenotype of these regulatory effector cells has been determined. Ly 7.2 was found to be selectively expressed on helper T cells; neither the suppressor cells nor the cytotoxic cells analyzed here were found to bear Ly 7.2 antigens. Mixing experiments demonstrated that the helper cell bears both Ly 1.1 and Ly 7.2. Removal of help by anti-Ly 7.2 treatment frequently enhanced suppressor activity. Suppressive activity was partially depleted by treatment with anti-Ly 6.1; helper cell activity was unaffected by such treatment. Cytotoxic T cell precursors bear neither Ly 6.1 nor Ly 7.2, but approximately half of the cytotoxic T cell effectors did bear Ly 6.1; none bore Ly 7.2. Analysis of populations depleted of 7.2-bearing cells should therefore allow the design of experiments that will increase our understanding of the interactions of various regulatory cell populations in the control of a cytotoxic T cell response.

Cytotoxic T cell responses are regulated by both helper cells and suppressor cells (1–9). We have developed *in vitro* systems to generate both alloantigen-specific helper T cells (4) and alloantigen-specific suppressor T cells (8). Low numbers of thymus cells are unable to generate a cytotoxic response in the absence of exogeneous helper activity when cultured with alloantigen; addition of antigen-specific helper T cells to these cultures results in the generation of strong cytotoxic responses (3–5). This system provides a measure of the helper activity that can be generated *in vitro*. If the number of thymocyte responder cells in culture is increased, or if splenic responder cells are used, a cytotoxic response is generated that can be suppressed by inhibitory cells generated *in vitro*. The use of these assay systems allows measurement of both helper and suppressor activity in a given cell population. We have used these techniques to determine the Ly phenotype of the cells that regulate the generation of cytotoxic cells. In a previous paper (10), we have shown that helper T cells, generated *in vitro*, have an Ly 1.1 $^+$ Ly 2.1 $^-$ phenotype. Suppressor cells, which are generated in the same cultures as helper cells, are of the Ly 1.1$^+$ 2.1$^+$ phenotype. Help and suppression are therefore mediated by physically distinct cell types.

In this report, we show that Ly 7.2 is selectively expressed on the effector T cells able to help a cytotoxic response; it is not expressed on suppressor or cytotoxic T cells. In all of these studies, we assay only the helper and suppressor activity that is resistant to the effects of a high dose of gamma irradiation, as has been discussed extensively (10). We have also analyzed the distribution of Ly 6.1 on the above described effector cells. We were able to demonstrate the presence of the Ly 6.1 marker on a cell that is involved in mediating the suppressive effect, and which may not be the same as the Ly 1$^+$2$^+$ or Ly 1$^-$2$^+$ suppressor cells previously described (8, 10). Cytotoxic T cells were found to be Ly 7.2-negative but a proportion of the killers bore Ly 6.1 in partial confirmation of a previous study in which the Ly 6.2 marker was used (11).

MATERIALS AND METHODS

Mice, culture methods, and the techniques to generate and assess helper and suppressor cell activity have been described (10; also see 4, 5, 8). Treatment with various anti-Ly sera and rabbit C have been described (10). Treated cell populations were never enriched; they were resuspended in the same volume of medium as were the normal mouse serum (NMS)[2] cells, and they were used as if at the NMS cell concentration (equivalent number). This means that fewer viable cells of the treated cells were added per culture, as compared to the NMS cultures. The functional assays described here provide internal controls for nonspecific cytotoxicity of the sera. Thus aliquots of the same treated populations were assayed in both helper and suppressor assays. Removal of one activity while another remains unaffected provides the most conclusive proof of serum specificity within a given treated population.

Antiserum	*Donor*	*Recipient*	*Serum Dilution(s) Used Here*
922 anti-Ly 6.1	C3H	(C3H.B6 × B6)F$_1$	1/10–1/20
754 anti-Ly 7.2	CXBk	(B6.C-H-2^d × CXBG)F$_1$	1/10–1/40

These same batches of serum, and frequently the same dilutions

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[2] Abbreviations used in this paper: NMS; normal mouse serum.

of serum, were used in the experiments reported by Shaw *et al.* (12)

RESULTS

Helper T cells bear Ly 7.2 antigens but do not bear Ly 6.1 antigen(s). Helper T cells can be generated by co-culture of CBA spleen cells with irradiated stimulator cells for 3 days *in vitro* (first step cells) (4). Helper activity is measured by the ability of first-step cells to collaborate with a low number of CBA thymocytes (3×10^5 per culture) in a response to irradiated BALB/c spleen cells in a second-step culture. First-step cells are always assayed at several cell numbers to allow quantitative comparison of the helper activity in various populations. To determine the Ly 6 and 7 phenotypes of these helpers, viable cells from 3-day first-step cultures were treated with anti-Ly sera plus rabbit C and then assayed for the degree of helper activity remaining after treatment. All treated populations were used at cell numbers equivalent to the NMS control, rather than equal numbers, to avoid disturbing the natural ratio of the unaffected cell types to one another. Each treated population was also assayed at a wide range of cell numbers to allow an assessment of the relative efficacy of each antiserum in removing activity. Since helper activity is not usually seen over the whole range of cell doses (4, 5, 10), this was important.

Treatment with anti-Ly 6.1 changed the pattern of helper activity as compared to NMS-treated cells with the result that the help was, if anything 3-fold more efficient (Table I, line 4, 1×10^3 first-step cells). This was in spite of the fact that Ly 6 killed approximately 90% of the viable first-step cells, In contrast, treatment of first-step cells with anti-Ly 7.2 completely removed helper activity at all cell numbers tested. This removal of helper activity did not represent a nonspecific cytotoxic effect since suppressor activity is unaffected by treatment with anti-Ly 7.2; similarly, cytotoxic T cell activity is unaffected by such treatment (see below).

Treated first-step cells that had not been irradiated were cultured for a further 5 days with fresh stimulator cells to assess the effect of treatment within the same population of treated cells on the generation of killer cells. Enhanced cytotoxicity was seen in anti-Ly 6.1-treated populations, whereas greatly reduced killer generation was observed in anti-Ly 7.2-treated cells (Table I, line 5). This probably reflects the dependence of further differentiation in culture upon Ly 7.2-bearing helper T cells, which will be discussed later.

Previous work has shown that the helper cell being measured in our assay system bears Ly 1.1 (10). The experiment of Figure 1 shows that the radioresistant helper cell, present in first-step cultures, that bears Ly 1.1 also bears Ly 7.2. Helper activity was completely removed by treatment with either anti-Ly 1.1 or anti-Ly 7.2 in this experiment. A mixture of these two treated and irradiated populations did not reconstitute the helper activity as measured in second-step cultures, suggesting that the same cell bears both markers.

Ly 6 and Ly 7 markers on suppressor cells. The enhanced generation of cytotoxicity in populations of cells treated with anti-Ly 6.1 suggested the existence of a suppressor cell bearing Ly 6 that was preventing the generation of killers (Table I, line 8, compare NMS and anti-Ly 6.1). To determine the Ly 6 and Ly 7 phenotype of the suppressor cells generated in first-step cultures, viable cells were harvested, treated with various antisera, irradiated, and assayed for activity in a second-step culture. In the experiment in Table II, suppression of both splenic and thymic cytotoxic T cell responses was measured.

Treatment with anti-Ly 6.1 reduced the level of suppressor

TABLE I

Ly 6.1 and Ly 7.2 on helper T cells generated in vitro[a]

Kind and No. of First-Step Cells		Treatment		
		NMS	Anti-Ly 6.1	Anti-Ly 7.2
		% specific lysis		
1. None	None	3 ± 1	3 ± 1	3 ± 1
2. CBA stimulated by BALB/c	1×10^4	25 ± 6	1 ± 1	5 ± 3
3. CBA stimulated by BALB/c	3×10^3	18 ± 7	11 ± 3	1 ± 1
4. CBA stimulated by BALB/c	1×10^3	6 ± 3	20 ± 6	6 ± 3
5. Unirradiated first-step cells[b]	1×10^5	14 ± 1	40 ± 3	1 ± 1

[a] First-step cultures consisted of 4×10^6 CBA spleen cells stimulated by 12×10^6 irradiated spleen cells for 3 days. Harvested first-step cells were treated with the indicated antiserum plus C, irradiated, and then assayed for helper activity in second-step cultures. Second-step cultures contained 3×10^5 CBA thymus cells plus 5×10^5 irradiated BALB/c spleen cells and the indicated kind and number of first-step cells. Each point represents the mean ± S. E. of 6 to 12 replicate cultures. Viability of first-step cultures after treatment: CBA stimulated by BALB/c NMS = 8.6×10^6 cells, anti-Ly 6.1 = 1.8×10^6 cells, anti-Ly 7.2 = 1.6×10^6 cells.

[b] 1×10^5 first-step cells were harvested, treated as indicated, and placed in culture again for 5 days with 5×10^5 irradiated BALB/c spleen cells. This method of assaying the effect of treatment has not been used in any other experiment reported here. The reduced helper activity by anti-Ly 6-treated cells seen in line 2 is most likely to be due to some variety of inhibitory effect since the helper activity appears at lower cell numbers. This phenomenon was not reproducibly observed.

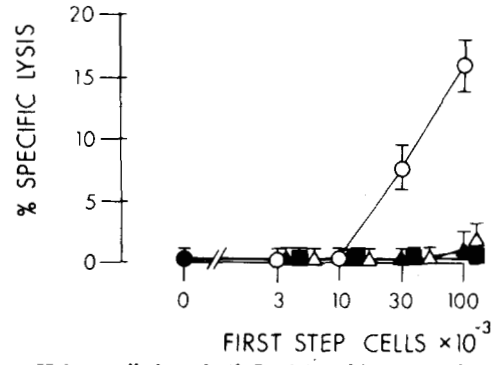

Figure 1. Helper cells bear both Ly 1.1 and Ly 7.2 antigens. 4×10^6 CBA spleen cells were cultured with 12×10^6 stimulator cells in Costar dishes for 3 days. Aliquots of the harvested viable cells were treated with the indicated antiserum plus rabbit C and then irradiated. The helper activity of these treated first-step cell populations was assayed in second-step cultures containing 1×10^5 CBA thymus cells and 5×10^5 irradiated BALB/c spleen cells. Each group consisted of 6 to 24 replicate cultures. Points are the mean ± S.E. ●, No first-step cells added; ○, first-step cells treated with NMS; ▲, first-step cells treated with anti-Ly 1.1; ■, first-step cells treated with anti-Ly 7.2; △, mixture of cells treated with anti-Ly 1.1 with cells treated with anti-Ly 7.2. Viability after treatment: NMS = 8.5×10^6 cells; anti-Ly 1.1 = 3.9×10^6 cells; anti-Ly 7.2 = 2.1×10^6 cells.

activity able to inhibit a response by splenic responders approximately 3-fold (Table I, A) such that 3×10^5 anti-Ly 6-treated cells were required to produce the level of inhibition seen with only 1×10^5 NMS-treated cells. Treatment with anti-Ly 7.2 had no effect on the suppressor activity as measured on splenic responder cells. When thymic responders were used (Table II, B), treatment of first-step cells with anti-Ly 6.1 reduced the effect of the suppressor activity only slightly and

treatment with anti-Ly 7.1 actually enhanced the supressive effect (Table II b, 3×10^4 first-step cells). This pattern was repeatedly observed. Similar results were obtained with suppressor cells harvested after 5 days of culture.

In Table III; the effects of anti-Ly 1.1 and anti-Ly 6.1 serum were compared by using a 3-day first-step population; Ly 1.1 is expressed on 3-day suppressor cells (10). Anti-Ly 1.1 treatment reduced the suppressive activity by 10-fold; whereas treatment with anti-Ly 6.1 reduced the activity by 3-fold when assessed for suppressive activity; thus 3×10^5 anti-Ly 1.1-treated cells, or 1×10^5 anti-Ly 6.1-treated cells, were required to give the same degree of suppression effected by only 3×10^4 NMS-treated cells. Mixtures of anti-Ly 1.1 and anti-Ly 6.1-treated populations yielded suppression that was better than that by the anti-Ly 6.1-treated cells alone. This could represent an additive effect of the suppression remaining in the two treated populations, or it could indicate that the cell bearing Ly 6.1 is different from the cell bearing Ly 1.1.

Ly 6 and Ly 7 markers on cytotoxic effector cells. Cytotoxic T cells were generated from splenic precursors in 5-day cultures. The viable cells from these cultures were treated with anti-Ly sera and assayed for cytotoxicity on P815 target cells. Anti-Ly 7 had no effect on cytotoxic T cells. Treatment with anti-Ly 6 removed about 50% of the cytotoxic cells (Fig. 2).

Ly 6 and Ly 7 markers on cytotoxic T cell precursors. We had repeatedly observed that in mixed lymphocyte cultures (MLC) that had been incubated for 3 days, harvested, treated with anti Ly 7.2, and cultured with fresh antigen for 5 more days, the cytotoxic activity was completely removed (Table I, line 8). This suggested that either the helper cells, the cytotoxic T cell precursors, or both were Ly 7.2-bearing cells. To type the Ly 7 phenotype of the killer precursor, normal spleen cells were treated with anti-Ly 7.2 and then cultured with stimulator cells for 5 days. In this case, the cytotoxicity generated was only moderately affected by the treatment, and was fully restored by the addition of helper cells to treated normal spleen cells, indicating that the cytotoxic precursor does not bear Ly 7.2 (Table IV). In other experiments, the cytotoxic activity was depressed to a much greater degree by treatment with anti-Ly 7.2, but in all cases the cytotoxic activity of the treated cells was fully reconstituted by addition of co-stimulator (12), a Con A-induced nonspecific stimulatory factor, or by irradiated first-step cells (Table IV; line 3).

TABLE II

Treatment of suppressor cells with anti-Ly 6.1 and with anti-Ly 7.2[a]

Treatment of First-Step Cells	None	No. First-Step Cells Added × 10^{-4}			
		30	10	3	1
		% specific lysis			
A. Suppression of a response by spleen cells					
1. NMS	64 ± 4	3	7 ± 1	43 ± 4	
2. Anti-Ly 6.1		5 ± 1	33 ± 3	54 ± 4	
3. Anti-Ly 7.2		5 ± 2	10 ± 2	44 ± 4	
B. Suppression of a response by thymus cells					
4. NMS	64 ± 5		0	27 ± 8	48 ± 5
5. Anti-Ly 6.2			10 ± 2	34 ± 9	67 ± 3
6. Anti-Ly 7.2			1	4 ± 1	23 ± 9

[a] 3×10^5 CBA spleen cells or 10×10^5 CBA thymus cells were cultured with stimulator cells and the indicated number and kind of 3-day first-step cells. Each group consisted of 4 to 12 replicate cultures: the values above represent the mean ± S.E.

TABLE III

Comparison of the effects of anti-Ly 1.1 treatment with those of anti-Ly 6.1 treatment of suppressor cells[a]

Treatment of First-Step Cells	None	No. First-Step Cells Added		
		30	10	3
		% specific lysis		
NMS	62 ± 1	2	5 ± 2	23 ± 2
Anti-Ly 1.1		18 ± 4	36 ± 6	56 ± 4
Anti-Ly 6.1		6 ± 2	18 ± 2	39 ± 1
Mixture of anti-Ly 1.1- and anti-Ly 6.1-treated cells		1	5 ± 2	36 ± 2

[a] See legend to Table IIA for culture conditions. Three-day first-step cells were used. The mixtures consisted of equal parts of anti-Ly 1.1- and anti-Ly 6.1-treated cells. Viability after treatment: NMS = 4×10^6 cells; anti-Ly 1.1 = 2.5×10^6 cells; anti-Ly 6.1 = 1.5×10^6. Values represent the mean ± S.E. of 4 to 12 replicate cultures.

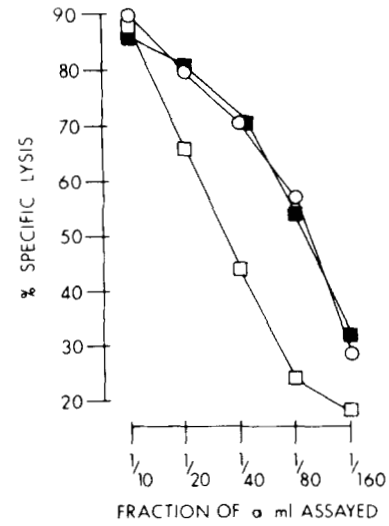

Figure 2. Ly 6.1 and Ly 7.2 markers on cytotoxic effector cells. Cytotoxic T cells were generated in cultures containing 3×10^5 CBA spleen cells and 5×10^5 irradiated BALB spleen. At day 5 of culture the cells were treated with the indicated serum plus C, and then assayed for cytotoxicity at cell numbers equivalent to the NMS control group. ○, NMS-treated cells, viability = 4.4×10^6; □, anti-Ly 6.1-treated cells, viability = 1.7×10^6; ■, anti-Ly 7.2-treated cells, viability = 3.3×10^6.

TABLE IV

Cytotoxic T cell precursors do not bear Ly 7.2 antigens[a]

Treatment	Fraction of a Culture Assayed		
	1/2	1/4	1/8
	% specific lysis		
NMS	96.7	75.7	60.9
Anti-Ly 7.2	84.1	66.4	39.9
Anti-Ly 7.2 + irradiated first-step cells	94.8	77.6	62.5

[a] 2×10^5 CBA spleen cells treated as indicated were cultured with 5×10^5 BALB/c stimulator cells for 5 days. In line 3, 1×10^4 irradiated first-step cells were added to the anti-Ly 7.2-treated precursors. Assay was on P815.

DISCUSSION

The distribution of Ly 6.1 and Ly 7.2 antigens on the cells that regulate the induction of cytotoxic T cell precursors has been determined. Helper T cells, previously shown to be Ly 1.1^+ Ly 2.1^- (10), were shown to be Ly 1.1^+, Ly 7.2^+, Ly 6.1^-. Of the T cell subsets typed here, only helper T cell effectors were

found to bear Ly 7.2 antigens. Mixing experiments were used to show that the Ly 1.1 marker and the Ly 7.2 marker are born by the same cell. Suppressor T cells, previously shown to be Ly 1.1^+ Ly 2.1^+, and a second subset of suppressors shown to be Ly 1.1^- Ly 2.1^+ (10), were found to be Ly 7.2-negative.

The generation of cytotoxic T cells by normal spleen cells treated with anti-Ly 7 was reduced to varying degrees in different experiments, suggesting that only a small proportion of the helper cells present in normal spleen (3) bear the Ly 7.2 marker. However, if cells that had been cultured with alloantigen for 3 days were treated with anti-Ly 7, irradiated, and added to second-step cultures, no helper activity could be detected implying that the majority of *in vitro* generated helpers do bear Ly 7.2. After a period in culture, the Ly 7^- helper cells in normal spleen had been almost completely replaced by Ly 7^+ helper cells. Since other work indicates that the helper T cell precursor itself does not bear Ly 7 (Pilarski and McKenzie, in preparation), it would appear that culture of spleen cells with antigen for 3 days allows differentiation of the helper activity from an Ly 7^- state to an Ly 7^+ state. The fact that in all experiments, some reduction in the generation of cytotoxic cells by normal spleen cells is observed upon treatment with anti-Ly 7 (see Table IV, and Reference 12) suggests that at least some proportion of the differentiated helper cells present in normal spleen (3) also bear Ly 7.2. This type of reasoning suggests that helper cells differentiate from an Ly 7^- precursor, to an Ly 7^- intermediate effector, and finally to an Ly 7^+ effector.

A second activity of Ly 7-bearing cells is implied by the fact that the suppressor activity of a culture containing thymocyte responders is frequently enhanced by treatment with anti-Ly 7 revealing previously undetected inhibitory potential. Since the only cell type found to bear Ly 7 in this system was the helper T cell effector, it appears that differentiated helper cells may exert an effect that is antagonistic to that of differentiated Ly 7^- suppressor T cells, thereby modulating suppressor effects. Finally, it seems significant that the T cell that is required for the production of a nonspecific Con A-induced stimulatory factor (co-stimulator) (12, 13) has a Ly phenotype identical to that of the helper effector cell described here.

The generation of cytotoxic T cells from normal spleen responder cells is partially affected by treatment with anti-Ly 6; this is not due to the presence of Ly 6 on the cytotoxic precursor (12, 14, and unpublished results). In contrast to the situation observed in the case of treatment of cultured cells with anti-Ly 7, treatment with anti-Ly 6 before a further period of culture causes a large increase in the generation of cytotoxicity. This implied the participation of a Ly 6-bearing inhibitor in the regulation of cytotoxic T cell generation. However, experiments designed to demonstrate more directly the expression of Ly 6 antigens by the suppressor cell were somewhat equivocal. In five out of seven experiments, treatment with anti-Ly 6 caused a 3-fold redution in the suppressor activity when the cells were assessed for their ability to suppress a response by cytotoxic cell precursors present in spleen. In parallel experiments, treatment with anti-Ly 1 or anti-Ly 2 caused a 10 to 30-fold reduction in suppressive activity (Table III and Reference 10). This observation raises some questions concerning the nature of the cell that bears Ly 6 and participates in the radioresistant suppression of a cytotoxic response. It is possible that the suppressor cell population is heterogeneous with regard to the expression of Ly 6 such that only some proportion (about 60%) of the suppressors are removed by treatment. Alternatively, perhaps a second cell type is required to participate in the suppression and it is this "accessory" cell that bears Ly 6.

It is interesting that the distribution of Ly 6 on cytotoxic cells is also heterogeneous. Unlike previous studies in which (11,12) anti-Ly 6.2 antiserum (11) was used, we find that approximately half of the alloantigen-specific cytotoxic T cells do not bear Ly 6. It is unlikely that this represents inefficient cytotoxicity of the serum since in many experiments 60 to 90% of the viable cells were killed by anti-Ly 6.1 plus C, and yet the suppressor or killer activity was only marginally reduced. Double treatment with antiserum did not increase the depletion of cytotoxic cells. This was also true of treatment with anti-Ly 7.2. Ly 6.1 and Ly 7.2 must therefore be present on some non-T cells since anti-Ly 1.1, anti-Ly 2.1, or anti-Θ generally kill only 30 to 50 % of the same cell populations. Other work has shown these markers to be on both T and non-T cells, although the Ly 6.2 specificity appears to be much more widely distributed on differentiated T cells than is observed here for the Ly 6.1 specificity suggesting that these two allelic specificities may be quite different. (11, 15–17, 19, 20). Preliminary experiments indicate that the antigen-bearing macrophage-like cells that induce specific T cell proliferation seem to bear both Ly 6.1 and Ly 7.2 antigens (K. C. Lee and I. F. McKenzie, personal communication). Perhaps the elimination of a cell of this type results in the decreased suppressor effect.

In conclusion, this work shows that Ly 7.2 is selectively expressed only on helper T cell effectors. The helper cell that expresses Ly 7.2 also expresses Ly 1.1. This observation thus reveals a means of selectively depleting populations of differentiated helper effector cells.

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