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The production of lymphokines by primary alloreactive T-cell clones: a co-ordinate analysis of 233 clones in seven lymphokine assays

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Summary. A total of 233 primary alloreactive T-cell clones have been tested for the production of interleukin-2 (IL-2), interleukin-3 (IL-3), immune (gamma) interferon (IFN) and granulocyte–macrophage colony-stimulating factor (CSF-2), B-cell growth factor I and II (BCGFI, BCGFII), and eosinophil differentiation factor (EDF). EDF was assayed by means of the eosinophil differentiation assay (EDA).

Two principal correlations were observed: IL-3 was shown to be the major lymphokine detected in the bone marrow proliferation assay (BMPA) used to detect CSF-2, and there was a high correlation between the EDA and BCGFII. Subsequent work has suggested that this latter correlation is because a single factor is responsible for both activities.

Apart from these two exceptions, and low level correlations probably due to the fact that different assays detect more than one lymphokine, there was no evidence for co-ordinate expression of lymphokines. There was a large variation in amounts of individual lymphokines produced. More clones produced multiple lymphokines than would be expected from independent control. Taken together, this pattern of regulation is consistent with the hypothesis that antigen stimulation of T cells results in the activation of all the lymphokine genes, but the amount of each produced is determined by secondary controlling mechanisms.

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INTRODUCTION

One fascinating aspect of T-cell biology is the wide range of different lymphokine activities produced by individual T-cell clones (Prystowsky *et al.*, 1982). Although many of these activities probably result from different biological properties of individual molecules, there undoubtedly remains a spectrum of different molecules produced. In this paper, we document an analysis of seven lymphokine activities in four panels of T-cell clones (totalling 233 clones). The purpose of this work has been an attempt to develop a model for the way in which the production of a spectrum of different lymphokines could be controlled.

For example, it has been claimed that interleukin-2 (IL-2) and colony-stimulating factor (CSF) (Watson 1983), as well as IL-2 and interleukin-3 (IL-3) (Miller & Stutman, 1983), are co-ordinately expressed. Co-ordinate control provides an economical model for the control of lymphokine secretion. As lymphokines (apart from IL-2, see Discussion) are produced more or less synchronously after stimulation of T cells (Ely *et al.*, 1981), it is a possibility that a single controlling system exists. If this is the case, then it would be expected that lymphokines would be co-ordinately expressed. However, the demonstration of co-ordinate expression would also be expected if a single molecule is detectable in two different assays. Thus, the identity of immune (gamma) interferon (IFN) as macrophage-activating factor (MAF) was first suggested by the fact

that they were co-ordinately expressed (Zlotnik *et al.*, 1983; Kelso & Glasebrook, 1984).

The present work extends this approach, and the assays used have been chosen because they allow quantification by automatic machines and because they detect characterized lymphokines. The assays tested firstly for four lymphokines which have been well characterized and gene-cloned: IL-2 (Fuse *et al.*, 1984), IL-3 (Fung *et al.*, 1984), IFN (Gray & Goeddel, 1983) and granulocyte–macrophage colony-stimulating factor (CSF-2) (Gough *et al.*, 1984) which, in these experiments, was measured by a bone marrow proliferation assay (BMPA) based on stimulation of DNA synthesis in bone marrow cultures (Horak *et al.*, 1983; Watson, 1983). Secondly, the assays tested for three lymphokines which have been partially purified and reasonably well characterized: B-cell growth factor I (BCGFI, BSF-1) (Howard, Nakanishi and Paul, 1984; Ohara & Paul, 1985), B-cell growth factor II (BCGFII, DL-BCGF-Swa) (Dutton, Wetzel & Swain, 1984), and eosinophil differentiation factor (EDF) which stimulates the production of eosinophils in bone marrow cultures (Sanderson, Warren & Strath, 1985). This eosinophil differentiation assay (EDA) was carried out by assaying for eosinophil peroxidase (Strath, Warren & Sanderson, 1985).

The approach was to isolate primary alloreactive T-cell clones in limiting dilution in the presence of a source of IL-2 (Glasebrook & Fitch, 1980). After 2 weeks in culture, the clones were restimulated with alloantigen in the absence of exogenous IL-2. The supernatants were collected and aliquots tested in each of the assays. The data were transferred to a computer and statistical analyses carried out.

MATERIALS AND METHODS

Isolation of T-cell clones

BALB/c.nimr and CBA/Ca.nimr mice were used. Cultures were established in round-bottomed microplates (Nunc, Copenhagen, Denmark). Responder spleen cells (100/well) were cultured with 2×10^5 irradiated stimulator cells and IL-2 (Sanderson & Strath, 1985) in a total volume of 100 μl of RPMI-1640 containing bicarbonate and supplemented with 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate and $7{\cdot}5 \times 10^{-5}$ M monothioglycerol (Sigma, Poole, Dorset) and 10% preselected fetal calf serum (Sera Labs, Crawley Down, Sussex). Irradiated spleen cells were obtained 1 hr after irradiating the mice with 1200 rads. IL-2 was obtained from the EL4 cell line (Farrar *et al.*, 1980) as described previously (Sanderson & Strath, 1985). After 10 days incubation at 37° in a humid atmosphere of 5% CO_2 in air, a further 100 μl of medium containing IL-2 was added to each well. After 14 days the cells in the wells were washed and restimulated with irradiated T-depleted stimulator cells in a total volume of 200 μl of medium without added IL-2. Spleen cells were T-depleted using monoclonal antibody NIM-R1 (Chayen & Parkhouse, 1982) and guinea-pig complement. After 24 hr (Experiment 4) or 40 hr (Experiments 1, 2 and 3), the supernatants were removed from wells showing T-cell growth for lymphokine assay.

Only T-cell colonies from plates where less than 20% of the wells showed growth were used. Thus, on the assumption of a Poisson distribution for the number of cells seeded in each well, less than 2% of the colonies would be expected to contain more than one clone.

Lymphokine assays

Each assay was carried out using 10 μl of T-clone supernatant in a total volume of 100 μl. For the EDA, the supernatant was diluted 1:10 before adding 10 μl to the wells. All assays were carried out in duplicate except the assay for EDA which was in triplicate. Round-bottomed microplates were used for all assays except for BCGF and IFN, where flat-bottomed microplates were used. DNA synthesis was determined in the IL-2, IL-3, BCGFII, BCGFI and BMPA by adding 0·5 μCi of [methyl-^{3}H]thymidine (5 Ci/mmol, Amersham International, Amersham, Bucks) for the last 4 hr of the incubation period. The cells were harvested onto glass fibre filter paper and processed for liquid scintillation counting. IL-2 was assayed on the CTLL cell line (Gillis *et al.*, 1978) and IL-3 on the 32D cell line (Greenberger *et al.*, 1983). Incubation time was 24 hr using 10^4 responder cells/well as described in detail previously (Sanderson & Strath, 1985). BCGFI was assayed on 5×10^4 splenic B cells in the presence of 5 μg/ml of affinity-purified goat anti-mouse immunoglobulin. B cells were obtained by T-depleting spleen cells of normal mice, followed by centrifugation through Percoll (Pharmacia, Milton Keynes, Bucks). Cells at the 75–85% interface were collected. Incubation time was 72 hr (Howard *et al.*, 1982). BCGFII was assayed on the BCL_1 cell line (Knapp *et al.*, 1979) as described by Dutton *et al.* (1984). The BCL_1 cells were obtained from the spleens of tumour-bearing mice after T-cell depletion. Cells were incubated for 72 hr at 2×10^4 cells/well.

The EDA was carried out as described (Strath *et al.*, 1985) using bone marrow cells from *Mesocestoides corti* infected mice (10^5 cells/well). After 5–6 days, the relative number of eosinophils was estimated by assaying for eosinophil peroxidase using the *o*-phenylenediamine hydrogen donor. Activity was read in a Titertek Multiscan at 492 nm. The BMPA was carried out in a similar way, except that DNA synthesis was determined after 60 hr (Horak *et al.*, 1983).

Interferon was assayed by inhibition of Semliki Forest virus-directed RNA synthesis in L929 fibroblasts, based on the method described by Allen & Giron (1970). The cells were distributed (4×10^4/well) and, after 18 hr, washed and incubated with clone supernatant in 100 μl of medium. After a further 18 hr, the medium was replaced with medium containing 3 μg/ml actinomycin D (Sigma) and 2×10^8 p.f.u./ml of Semliki Forest virus. After 2·5 hr, 0·5 μCi of [5,6-^{3}H]uridine (40–69 Ci/mmol, Amersham International) was added and the cells harvested after a further 2·5 hr, as described above. Interferon activity was expressed as the percentage inhibition of virus-dependent [^{3}H]uridine uptake.

Statistical analysis

The data from each assay were transferred to a computer file, and the means of replicate determinations of lymphokine activity for each clone were calculated. Replicates that were judged so different that the mean could not be relied upon were noted. A clone was scored as negative for a particular lymphokine activity if its mean was less than the control mean plus three standard deviations. Clones negative for all lymphokine activities, as well as clones giving unreliable data (see above), were omitted from further analyses.

The association between expression of different lymphokine activities was first examined by plotting scatter diagrams of the activity levels in the individual clones for each pair of lymphokines (for example see Figs 1 and 2). For each scatter diagram, a correlation coefficient was calculated to measure the degree of linear association between the two lymphokine activity levels. These correlation coefficients were referred to standard tables (e.g. Lindley & Scott, 1984) to determine their levels of statistical significance.

The possibilities of further analyses by (i) converting the primary assay responses into units of lymphokine activity through reference to dilution curves for standard lymphokine preparations, and (ii) attempting to fit functional relationships to the data in the scatter diagrams, were investigated. In view of the wide interclonal variations (see Figs 1 and 2), however, these analyses did not reveal any important information additional to the correlation analyses, and they are not presented here.

RESULTS

Table 1 summarizes the number of clones producing detectable lymphokine activity (greater than the control mean plus three standard deviations). These data were collated after eliminating clones negative in all assays (55 clones) and clones containing unreliable data (23 clones). In Experiments 1, 2 and 3, clones were scored positive for growth before restimulation. Thus, many of the negative clones may have been antigen non-specific, that is, T cells expanded by IL-2 and independent of antigen. In Experiment 4 the clones were maintained in IL-2 and scored for growth after 3 days. Under these conditions, antigen non-specific clones die during the restimulation period without IL-2. In this experiment, only three out of 90 clones were negative in all assays.

Less than 20% of the clones were positive for IL-2, and the levels of IL-2 detected represented less than 10% of the activity produced by the EL4 line. Similarly, less than 10% of the clones were positive for IFN, although a concanavalin A (Con A)-stimulated spleen supernatant used as a positive control gave detectable activity at a concentration of 2% in each experiment. In contrast, a high proportion of clones produced IL-3.

Table 2 gives the correlation coefficients for each pair of different lymphokine activities in each experiment. Not all assays were carried out in each experiment. Only a representative part of these data are presented graphically. Figure 1 shows data from Experiment 4, and Fig. 2 shows data from Experiment 2. The only correlation not shown graphically is that between IL-2 and IFN where the numbers positive were very low and the correlation not significant in each of the two experiments.

Correlations with IL-2

In the comparison with IL-3, two out of four experiments gave significant correlations ($P < 0{\cdot}01$), but with correlation coefficients of less than 0·5. Inspection of the data (Fig. 1a) shows a tendency for IL-2 high positives to produce high IL-3 levels. However, many

Table 1. Summary of clones positive in each lymphokine assay

Exp.	Total*	IL-2	IL-3	BCGFI	BCGFII	EDA	BMPA	IFN	Alloreactivity
1	65	5	41	34	27	NT†	27	4	CBA∝BALB/c
2	40	3	31	13	NT	17	31	5	CBA∝BALB/c
3	41	4	38	16	12	12	29	NT	BALB/c∝CBA
4	87	27	87	41	63	80	NT	NT	BALB/c∝CBA
Total	233	39/233	197/233	104/233	102/193	109/168	87/146	9/105	
% positive		17	85	45	53	65	60	9	

Clones scored positive if higher than control mean + 3 SD.
* Total does not include clones negative in all lymphokine assays.
† NT, not tested.

Table 2. Correlation coefficients describing the associations between different lymphokine activities

	Exp.	IL-3	BCGFI	BCGFII	EDA	BMPA	IFN
IL-2	1	0·21	0·16	0·50**	NT†	0·22	−0·30
	2	0·49*	0·35	NT	0·44**	0·45**	0·24
	3	0·18	0·09	0·51**	0·50**	0·53**	NT
	4	0·33*	0·23	0·38**	0·26	NT	NT
IL-3	1		0·49**	0·04	NT	0·72**	−0·04
	2		0·12	NT	0·72**	0·92**	0·40
	3		0·29	0·45*	0·48*	0·75**	NT
	4		0·30*	0·27	0·34	NT	NT
BCGFI	1			0·36*	NT	0·47**	0·00
	2			NT	0·31	0·11	−0·20
	3			0·29	0·24	0·30	NT
	4			0·36**	0·36**	NT	NT
BCGFII	1				NT	0·12	−0·20
	2				NT	−0·28	−0·34
	3				0·77*	0·58**	NT
	4				0·68**	NT	NT
EDA	1					NT	NT
	2					0·63**	0·09
	3					0·75**	NT
	4					NT	NT
BMPA	1						0·13
	2						0·50**
	3						NT
	4						NT

The numbers of clones on which these correlation coefficients were based were 65, 40, 41 and 87 for Experiments 1, 2, 3 and 4, respectively (see Table 1).
* Statistically significant at 1% level ($P < 0·01$).
** Statistically significant at 0·1% level ($P < 0·001$).
† NT, not tested.

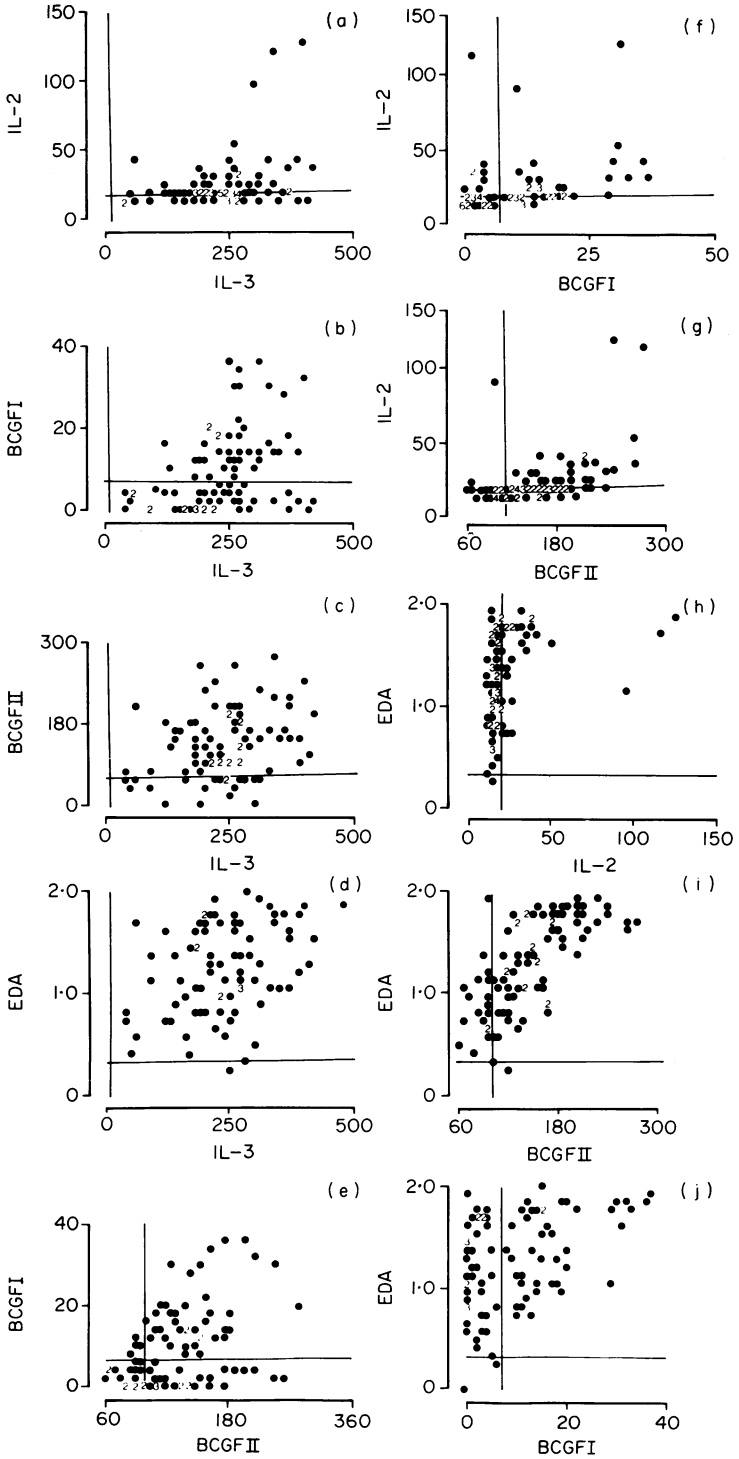

Figure 1. Scatter diagrams for pairs of different lymphokine activities in a panel of 87 T-cell clones (Experiment 4, see Tables 1 and 2). EDA is presented as optical density (A_{492}), all the other assays are in [H]TdR uptake (c.p.s.). The horizontal and vertical lines show the control means + three SD for each assay. Each dot represents a single clone, and numerals indicate the number of clones plotting over a single point.

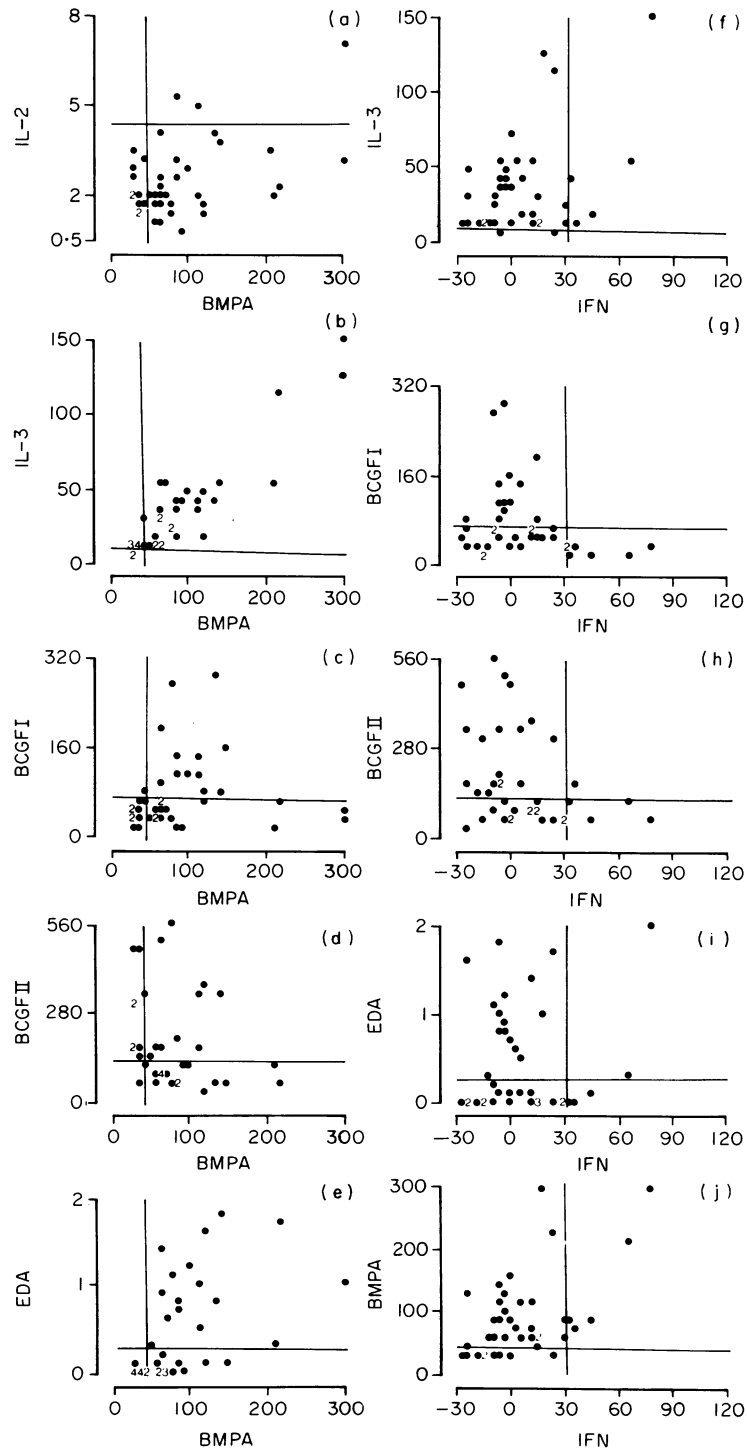

Figure 2. Scattter diagrams for pairs of different lymphokine activities in a panel of 46 T-cell clones (Experiment 2, see tables 1 and 2). Details as in Fig. 1, except that IFN is expressed as the percentage inhibition of viral-dependent [^{3}H]uridine uptake. Not all pairs of different lymphokine activities from Experiment 2 are represented in the figure.

high IL-3 producers were negative for IL-2, so there is clearly no evidence for co-ordinate expression of these two lymphokines.

There was no significant correlation between IL-2 and BCGFI. Figure 1f shows a complete scatter of the two activities. On the other hand, the correlation between IL-2 and BCGFII was highly significant ($P < 0{\cdot}001$) in each of three experiments, although the individual correlation coefficients were 0·51 or less. Figure 1g shows, however, that a large number of clones were negative for one or both activities, so the correlations, although statistically significant, appear not to be very important biologically. This was also the case in Experiments 1 and 3 (data not shown). A very similar effect was seen in the relationships of IL-2 with the EDA and the BMPA, where analysis revealed significant correlations ($P < 0{\cdot}01$) in two out of three experiments, but where the plotted data made it clear that no important association between these lymphokine activities existed (Fig. 1h and Fig. 2a, respectively).

Neither of two experiments suggested any correlation between IL-2 and IFN, with clones positive for one activity being negative for the other in each case.

Correlations with IL-3

In two out of four experiments with BCGFI and one out of three with BCGFII, significant correlations with IL-3 were found. However, in each case the correlation coefficient was less than 0·5 and the scatter (see Fig. 1b and c) was quite wide.

For the relationship between IL-3 and EDA, all three experiments showed significant correlation, suggesting a tendency for high IL-3 producers to be highly positive in the EDA (Fig. 1d), although again the scatter was wide.

The association between IL-3 and the BMPA was highly significant ($P < 0{\cdot}001$) in each case, with all correlation coefficients greater than 0·7. The plotted data show a clear correlation (Fig. 2b). There was no suggestion of a relationship between IL-3 and IFN (Fig. 2f).

Correlations with BCGFI

Two out of three experiments revealed significant correlations between BCGFI and BCGFII, although the correlation coefficients were less than 0·4 in each case. The data showed that clones highly positive for BCGFI were all positive for BCGFII (Fig. 1e), although the reverse was not the case.

One of the three experiments suggested a correlation of BCGFI with the BMPA, though this appeared to be due to a high proportion of clones giving low activity in both assays in this particular experiment. Figure 2c clearly shows the lack of correlation between these activities. Neither of two experiments suggested any correlation between BCGFI and IFN.

Correlations with BCGFII

Both experiments showed a highly significant ($P < 0{\cdot}001$) correlation between BCGFII and the EDA. Inspection of the data (Fig. 1i) showed a clear relationship.

One experiment indicated a correlation between BCGFII and the BMPA, but again this appeared to be due to a high proportion of clones giving low activity in both assays in this experiment. The data shown in Fig. 2d are representative of the other two experiments, and showed no evidence of a correlation. Similarly, there was no correlation between BCGFII and IFN (Fig. 2h).

Correlations with EDA

The high correlation between the EDA and BCGFII is discussed above. Two experiments also revealed a highly significant correlation ($P < 0{\cdot}001$) between the EDA and the BMPA. The data in both experiments indicated that clones positive in the EDA were positive in the BMPA, although the reverse was not the case (Fig. 2e). There was no correlation between the EDA and IFN in a single comparison (Fig. 2i).

Correlation with BMPA

The relationship between the BMPA and IFN was significant in one out of two experiments, but this appeared to be due to the high proportion of clones negative for both activities. Figure 2j indicates that clones highly positive in the BMPA showed a scatter of IFN activities.

Frequency of multiple producers

The data from Experiment 4 were analysed to determine the number of clones producing different numbers of lymphokines. Only data for IL-2, IL-3, BCGFI and BCGFII were included as these were known to be different factors. Table 3 shows that more clones produced either low or high numbers (0, 1 or 4)

Table 3. Frequency of clones producing multiple lymphokines (data from Experiment 4, Table 1)

Number of lymphokines produced	Number of clones		Differences (0 − E)
	Observed	Expected*	
0	3	0·3	2·7
1	15	11·0	4·0
2	27	36·9	−9·9
3	23	33·2	−10·2
4	22	8·3	13·7
Total	90	89·7†	

* Calculated number of clones producing 0, 1, 2, 3 or 4 lymphokines, assuming independent control. These numbers were calculated from the proportions of clones positive for each of the four lymphokines (IL-2, IL-3, BCGFI, BCGFII) which were known to be different factors. If these proportions are denoted p, p, p, p, then, for example, the expected number of clones producing one lymphokine is $[p_1(1-p_2)\ (1-p_3)\ (1-p_4)+(1-p_1)p_2(1-p_3)\ (1-p_4)+(1-p_1)\ (1-p_2)p_3(1-p_4)+(1-p_1)\ (1-p_2)\ (1-p_3)p_4] \times 90$. Expressions for the other expected number of clones were similarly derived by summing permutations of probability terms giving the requisite numbers of lymphokines produced.

† Difference from 90 due to rounding error.

of lymphokines than would be expected from independent control, while fewer clones produced intermediate numbers (2 or 3).

DISCUSSION

The lymphokine assays chosen for this study were all reported to detect characterized lymphokines, and were amenable to automated analysis which eliminated subjectivity. The only data selection applied was when replicates were so wide apart that it was unclear whether the clone was a non-producer or a high producer. In these cases, all the data from that clone were eliminated from the data file. Apart from this, clones negative in all the assays were eliminated. The latter would include clones incorrectly scored for growth, clones that were not T cells, and presumably any T cells which did not produce detectable levels of lymphokines. It should be noted that in Experiment 4, where growth was scored after restimulation, only three out of 90 were negative in all assays. This indicates that very few, if any, alloreactive T cells produce no lymphokine.

One limitation in this type of analysis is that some assays detect more than one lymphokine, so that there is a tendency towards apparent co-ordinate expression of two activities, when the two lymphokines primarily detected by two assays are not in fact co-ordinately expressed. A number of low level correlations were observed which fit this explanation (see below).

The relationship between lymphokine production and Lyt-2 phenotype has been investigated in detail by Kelso & MacDonald (1982). They found that both Lyt-2$^+$ and Lyt-2$^-$ populations produces MAF and CSF-2. On the other hand, IL-2 was only detected in the Lyt-2$^-$ clones. Thus, with the possible exception of IL-2 (and the problems of interpretation of these data are discussed below), it seems unlikely that T cells can be divided into lymphokine producers and non-producers, although there is a tendency or Lyt-2$^-$ T cells to produce higher levels than Lyt-2$^+$ T cells (Kelso & Glasebrook, 1984).

Two remarkable correlations appeared in this analysis. Firstly, there was a good correlation between IL-3 and the BMPA. Subsequent work has shown that purified IL-3 is active in this assay, and so this suggested that IL-3 is the major lymphokine detected by the BMPA. This, in turn, implies that T cells produce only low levels of CSF-2 compared to their IL-3 activity. Secondly, there was a good correlation between the EDA and BCGF II. This was entirely unexpected, and subsequent work has shown that the two activities copurify, thus suggesting that a single factor mediates both activities (Sanderson *et al.*, 1985).

The results with IL-2 are difficult to interpret as the amount of IL-2 detected represents the balance between production and consumption of IL-2 by the T clones, so that this assay gives little insight into the levels of production. Although some other correlations were observed, so that the high IL-2 producers tended to produce high levels of other lymphokines as well, these could not be attributed to any significant co-ordination (see results). These results probably reflect the known capacity of IL-2 to enhance the production of other lymphokines (Kelso & Glasebrook, 1984; Kelso *et al.*, 1984).

Only relatively few clones produced IFN. This may be attributed to the lack of exogenous IL-2 during stimulation, as IL-2 is known to enhance the release of IFN (Kelso *et al.*, 1984). However, in no case was there evidence of co-ordinate expression with any other lymphokine.

In general, some low correlations were observed between different haemopoietic assays. This appears

to be due to multiple activities of individual factors in the different assay systems. For example, IL-3 induces some eosinophil differentiation (Sanderson *et al.*, 1985), which probably explains the low correlation between IL-3 and the EDA. Similarly, there was a correlation between the EDA and the BMPA, presumably due to the stimulation of DNA synthesis during eosinophil differentiation.

There was a clear tendency for clones producing high levels of BCGFII to be positive for BCGFI. This was even more marked in the comparison between BCGFI and the EDA (Fig. 1j). This suggests the possibility that the factor active in the BCGFII assay (which also appears to be responsible for eosinophil differentiation) may also have some activity in the BCGFI assay.

Apart from these interactions, it would appear from these data that, when assays detect a single lymphokine, there is no correlation between the amounts of different lymphokines produced by individual clones. Furthermore, the data indicate that in a panel of T clones there is a range of levels of activity for each lymphokine, from undetectable to high. When clones were grouped according to the number of lymphokines detected, it was found that more clones produced low or high, rather than intermediate, numbers of lymphokines than would be expected from independent control. Clearly, increasing the sensitivity of the assays would enhance this effect.

Lymphokine production is induced by antigen stimulation of the T cell and continues for about 24 hr before production is closed down (Ely *et al.*, 1981). It appears that, once established, the pattern of lymphokine production remains constant, so that each clone retains the same profile of lymphokine production with continued culture (Kelso *et al.*, 1984; C. J. Sanderson, unpublished observations). The experiments reported in this paper were carried out to shed some light on the type of control mechanism that might be involved in the induction of lymphokine production.

Clearly, the lymphokines are not co-ordinately *expressed*. However: (ii) they do not appear to be independently controlled, (ii) there is a wide variation in amounts of individual lymphokines produced by different clones, and (iii) the proportion of positive producers would almost certainly be increased if more sensitive assays were used. Therefore, it is not unreasonable to suggest that all lymphokine genes are activated by antigen stimulation. Thus, they may well be co-ordinately *controlled*, but secondary controlling mechanisms determine the amount of each lymphokine produced. Analysis of this control at the molecular level might reveal both a system for co-ordinate activation, as well as a system for independently controlling the subsequent expression of each of these genes. This latter system must establish and maintain the profile of lymphokines produced by individual clones.

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