

# Localization of $\gamma/\delta$ T Cells to the Intestinal Epithelium Is Independent of Normal Microbial Colonization

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## Summary

Using monoclonal antibodies identifying all  $\gamma/\delta$  and  $\alpha/\beta$  T cell receptors in cytofluorometric analysis, we have compared the composition of intestinal intraepithelial lymphocytes (i-IEL) in euthymic and athymic germ-free (GF) and conventional (SPF) mice. The results show a marked influence of microbial colonization in the numbers of single-positive ( $CD4^+$  or  $CD8^+$ )  $\alpha/\beta$  i-IEL, but little effect in the pool size or characteristics of  $\gamma/\delta$  i-IEL. In young athymic mice, virtually no  $\alpha/\beta$  i-IEL are detected, while considerable numbers of  $\gamma/\delta$  i-IEL remain, though reduced in GF animals.

$\gamma/\delta$  T cells are relatively rare in the lymphoid organs of adult mice (1–5), but well represented in epithelia (5–10). The biological roles of these lymphocytes are unknown, but their preferential localization at the body surfaces has led to suggestions of a defensive role directed against microbial invasion (11). On the other hand, their skewed receptor repertoires and limited diversity at several anatomical sites (9, 10, 12–14) indicate that specific ligands for  $\gamma/\delta$  TCR are self components and that intraepithelial localization may be (external) antigen-independent (15). In apparent contrast,  $\alpha/\beta$  T cells recirculate and are selectively retained in the sites where foreign antigen is presented (16, 17). The comparison of  $\gamma/\delta$  and  $\alpha/\beta$  T cell populations in epithelia of animals that are either normal or free of microbial colonization should be informative in this regard. Intraepithelial lymphocytes (i-IEL)<sup>1</sup> in the mouse small intestine contain a subset of  $\gamma/\delta$  cells (7, 18) with characteristic V gene usage and receptor diversity (10, 12, 13, 19, 20). Using an anti-C $\delta$  monoclonal antibody identifying all  $\gamma/\delta$  T cells (5), we have now compared i-IELs in germ-free (GF) and specific pathogen-free (SPF) adult mice, and found that colonization by the normal intestinal flora has little effect on either the numbers or the phenotype of  $\gamma/\delta$  cells. In contrast, the i-IEL  $\alpha/\beta$  T cell pool is sharply reduced in GF mice, particularly the single positive ( $CD4^+$  or  $CD8^+$ ) cells. These results indicate a predominant role of  $\alpha/\beta$  T cells in the response to microorganisms of the intestinal mucosa, and thus suggest other roles for  $\gamma/\delta$  cells.

## Materials and Methods

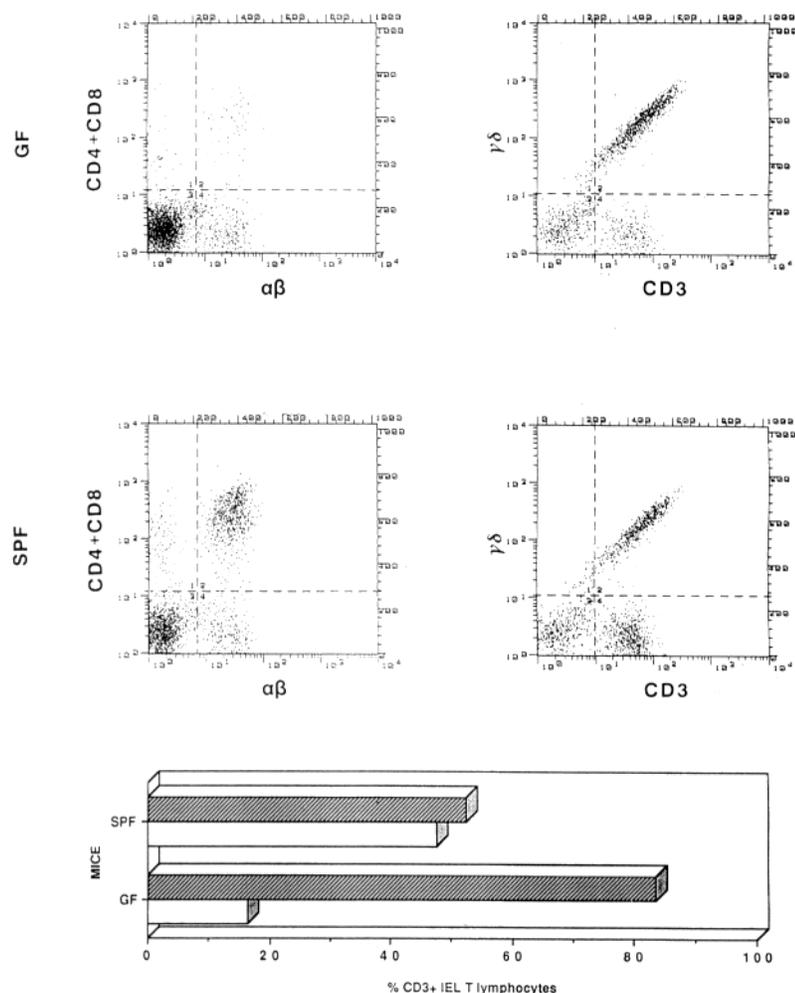
**Mice.** GF BALB/c animals are from a stock maintained under germ-free conditions over 40 generations at Ciba-Geigy AG, Basel, Switzerland. All GF mice studied here were 7–14 wk old and devoid of macroscopically detectable lymph nodes and Peyer's patches. SPF BALB/c mice were from the same animal facilities, age and sex matched with GF animals.

**Monoclonal Antibodies.** The following mAbs were used: 3A10, anti C $\delta$  (5); H57-597, anti C $\beta$  (21); 145-2C11, anti-CD3 (22); J.1.J, anti-Thy-1.2 (23); GK-1.5, anti-CD4 (24); 53.6.72, anti-CD8 (25).

**Lymphoid Cell Preparations.** i-IEL cell suspensions were prepared according to standard procedures (26–28) with some minor modifications (Mota-Santos, T., et al., manuscript in preparation). Briefly, small intestines free of mesenterium were removed into PBS, and extensively flushed to eliminate the lumen content. Peyer's patches were removed, and the intestines were longitudinally opened and cut in fragments of 1–2 cm long. These were gently shaken five consecutive times in Ca and Mg-free (CMF) HBSS. Fragments were then transferred to 50-ml tubes (Falcon Labware, Oxnard, CA) and incubated for 7–10 min in 25 ml CMF containing 5 mM EDTA and 70 mg/ml of DTT, at room temperature (RT) with gentle stirring (100 rpm). Fragments were then let sediment for 5 min on ice and the cell-containing supernatant recovered. i-IEL cells were washed three times and passed through a nylon mesh and siliconized glass wool columns to remove aggregates and dead cells. In the present experiments no further purification steps, namely Percoll gradients, were used. Total live cells were scored by trypan blue exclusion, and fluorescence stainings were performed. Spleen cells were washed and suspended in fluorescence medium: BSS (balanced salt solution) without red phenol, containing 3% FCS and sodium azide (0.015 M).

**Immunofluorescence and FACS Analysis.** Immunofluorescence stainings have been described before (29). In short,  $1\text{--}1.5 \times 10^6$

<sup>1</sup> Abbreviations used in this paper: CMF, Ca and Mg-free; GF, germ-free; i-IEL, intraepithelial lymphocytes; SPF, specific pathogen-free.



**Figure 1.** FACS analysis of i-IEL from 7-wk-old SPF and GF BALB/c mice, as indicated. The lower panel represents distributions of  $\alpha/\beta$  (open bars) and  $\gamma/\delta$  (shaded bars) within the CD3<sup>+</sup> T lymphocyte pool. Very similar results were obtained in a total of six animals analyzed in each group.

**Table 1.** Numbers of CD3<sup>+</sup> Intestinal Intraepithelial Lymphocytes Recovered from GF or SPF Animals

Exp. Animal	Number of CD3 <sup>+</sup>	CD8	Thy-1*
	$\times 10^{-6}$	%	%
1 SPF	7.2	ND	34.9
GF	6.9	ND	9.0
2 SPF	3.7	89	ND
GF	1.9	95	ND
3 SPF	6.0	ND	33.3
GF	3.4	ND	6.0
4 SPF	ND	83	ND
GF	2.3	85	ND

\* Percentages refer to total CD3<sup>+</sup> cells.

cells were incubated in microtiter plates for 20 min on ice with biotinylated (30) or FITC-labeled mAbs, and washed three times with fluorescence medium, before addition of streptavidine-phycoerythrin (PE), (Becton Dickinson & Co., Mountain View, CA) together with FITC-labeled antibodies. In single, as well as double stainings, propidium iodide was added.

Flow cytometric analysis was done in a FACScan (Becton Dickinson & Co.). In every case, dead cells were eliminated with an acquisition gate correlating FL1 (PE) and FL3 (PI), since dead cells, but not PE<sup>+</sup> live cells, always appear in the diagonal of this dot plot; in addition, a light scatter (FSC/SSC) gate removed red cells and debris. Single stainings for some lymphocyte markers in the total cell population were carried out in each experiment with conditions of acquisition, where intestinal epithelial cells were also scored, allowing the determination of absolute numbers per intestine of the respective lymphocyte population. In these cases, 17,000 events were acquired per sample. For double stainings, the FSC/SSC gate was constructed following the normal distribution of lymphocytes for these two parameters, while the FL1/FL3 gate excluded autofluorescence, diagonally positioned, intestinal epithelial cells. All gates were controlled before acquisition using anti-CD3-FITC staining of total i-IEL cell preparations.

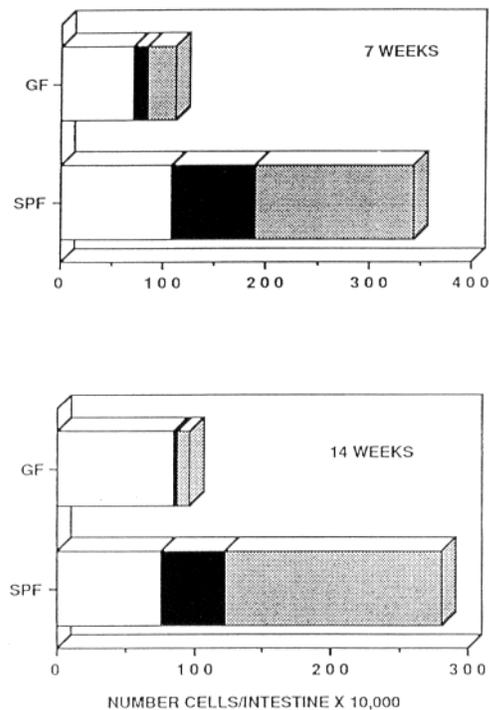
**Table 2.** Subpopulations of Intestinal Intraepithelial and Splenic Lymphocytes in GF and SPF Mice

Exp.	i-IEL	Number of cells/organ	
		SPF	GF
$\times 10^{-5}$			
1	$\gamma/\delta$	37.8 $\pm$ 0.1	57.4 $\pm$ 6.6
	$\alpha/\beta$ Total	34.3 $\pm$ 2.1	11.3 $\pm$ 1.6
	CD4 <sup>+</sup>	8.0 $\pm$ 1.2	1.3 $\pm$ 0.3
	CD8 <sup>+</sup>	15.6 $\pm$ 0.8	2.8 $\pm$ 0.3
	DN	10.8 $\pm$ 0.2	7.1 $\pm$ 0.8
2	$\gamma/\delta$	31.9 $\pm$ 13.5	25.5 $\pm$ 3.4
	$\alpha/\beta$ Total	28.1 $\pm$ 12.8	9.5 $\pm$ 3.4
	CD4 <sup>+</sup>	4.6 $\pm$ 1.8	0.3 $\pm$ 0.1
	CD8 <sup>+</sup>	15.9 $\pm$ 8.2	0.8 $\pm$ 0.2
	DN	7.6 $\pm$ 3.3	8.5 $\pm$ 3.4
$\times 10^{-6}$			
Spleen			
	CD3 <sup>+</sup>	44.3 $\pm$ 2.8	26.0 $\pm$ 8.3
	CD4 <sup>+</sup>	25.9 $\pm$ 3.4	15.7 $\pm$ 4.7
	CD8 <sup>+</sup>	11.6 $\pm$ 0.8	7.4 $\pm$ 2.1
	IgM <sup>+</sup>	44.1 $\pm$ 7.7	28.4 $\pm$ 8.9

The results are means and SD obtained from the individual analysis of three mice per group, at 7 (Exp. 1) and 14 wk (Exp. 2) of age. Total cell numbers were calculated from CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells scored in single stainings and acquisition gates that included total live cells in the preparations (17,000 events per sample). These numbers were thereafter used to calculate the pool size of each subpopulation, together with the respective frequencies obtained from double stainings for  $\gamma/\delta$  or  $\alpha/\beta$  and CD3, CD4, or CD8 in acquisition gates where epithelial cells were now eliminated (6,000–8,000 events) (see Materials and Methods).

## Results and Discussion

We have analyzed i-IELs by two-color cytofluorometry, using anti-CD3 and either anti-C $\delta$  (5) or anti-C $\beta$  (21) antibodies, which allow the identification of all T cells. As shown in Fig. 1, SPF and GF animals differ markedly in the contribution of each T cell subset to the i-IEL CD3<sup>+</sup> lymphoid population. In GF mice, the proportion of  $\alpha/\beta$  T cells in i-IEL (17%, range 15–22%) is one-third of that in i-IEL from SPF mice (average 48%, range 30–60%). The increased representation of  $\gamma/\delta$  T cells in GF mice (84% vs. 52% in SPF), however, could be relative and both cell types be actually depleted by microbial deprivation. Absolute numbers of intra-epithelial lymphocytes recovered from the intestinal wall were therefore determined and are shown in Table 1. No great difference between SPF and GF animals was observed as to the total number of CD3<sup>+</sup> T cells in several independent ex-

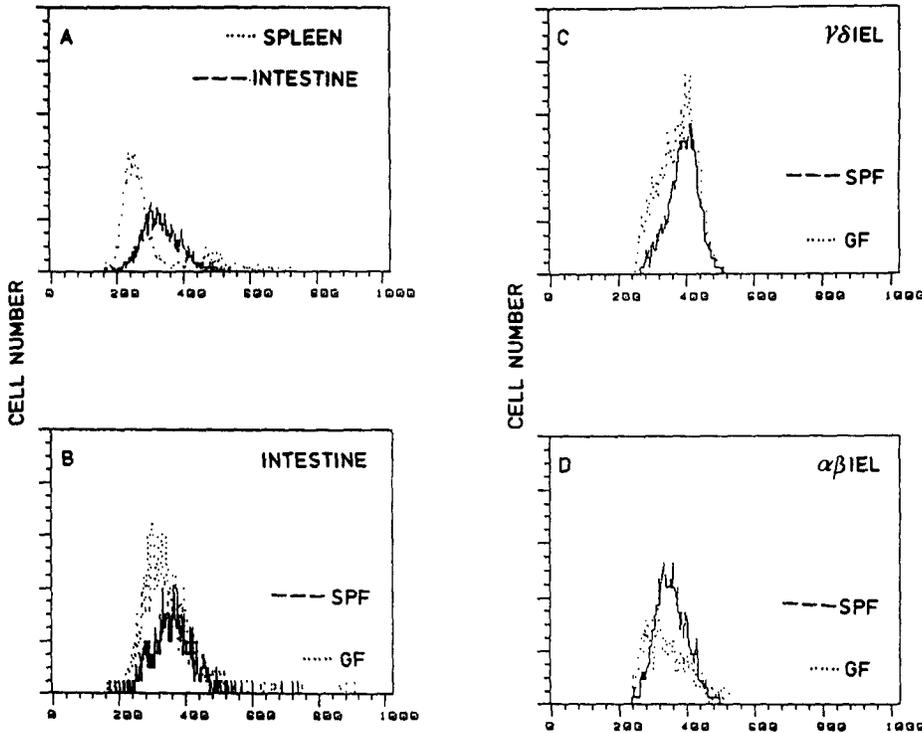


**Figure 2.** Absolute numbers of single positive (CD4, closed bars, or CD8, shaded bars), and CD4<sup>+</sup>CD8<sup>−</sup> cells (open bars)  $\alpha/\beta$  T cell i-IEL in GF and SPF mice, studied by two-color analyses at 7 and 14 wk of age. The data represent the mean of three independently studied animals in each group.

periments, indicating that  $\gamma/\delta$  T cells may actually increase in GF mice, as compared with age-matched SPF animals. Table 2 shows that this is the case in relatively young animals (7 wk), while in older individuals, there is no significant difference between GF and SPF conditions. In contrast, the single positive (CD4<sup>+</sup> or CD8<sup>+</sup>)  $\alpha/\beta$  subset in GF mice is 6–20-fold lower than in SPF mice. It appears, therefore, that lower i-IEL recoveries in GF animals are essentially the result of marked decreases in  $\alpha/\beta$  T cell colonization, while  $\gamma/\delta$  cells are better represented in GF than in SPF mice. Curiously, double-negative  $\alpha/\beta$  T cells are barely reduced and constitute the major subpopulation within this cell type GF animals (Table 2 and Fig. 2). Though less marked, the decrease in  $\alpha/\beta$  T cell numbers is also apparent in the spleen of GF mice, which accounts for 30% fewer T cells in the organ (Table 2). No significant age-related modifications of these patterns were observed from 7 to 14 wk of age.

We next evaluated parameters of cell activation in i-IEL populations from GF and SPF mice. First, cell size distributions (assessed by FCS) of  $\gamma/\delta$  i-IEL were very similar in the two groups of animals. Since the i-IEL CD3<sup>+</sup> cells are significantly larger than the splenic CD3<sup>+</sup> population, this indicates that even in GF animals intestinal T cells are indeed activated (Fig. 3). This conclusion applies to  $\alpha/\beta$  i-IEL as well, since there is also little difference in the mean size of  $\alpha/\beta$  T cells in GF and SPF mice. Second, the fraction of  $\gamma/\delta$  cells expressing surface CD8 molecules was not different

## SIZE OF CD3<sup>+</sup> CELLS

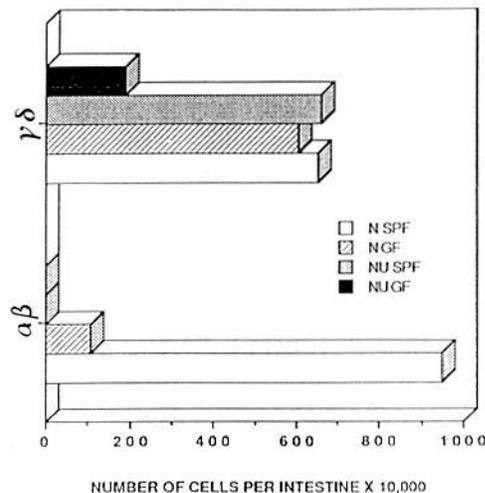


**Figure 3.** Forward light scatter (FSC) profiles of  $\alpha/\beta$  and  $\gamma/\delta$  i-IEL and splenic T cells, as indicated. Overlapped curves in each panel were compared using Kolmogoroff-Smirnov statistics. No significant difference was observed for the curves represented in B, C, and D, while the FCS profiles of splenic and i-IEL CD3<sup>+</sup> cells shown in A were different; the null hypothesis is rejected with a probability lower than  $p < 0.001$  (given  $D = 0.48$  and  $D/s(n) = 14.13$ ).

in GF and SPF animals (see Table 1). This is particularly interesting since CD8 expression may be correlated with activation in this lymphocyte subset (31, 32). Finally, IL-2 receptor expression by CD3<sup>+</sup> i-IELs was monitored but found negative in both groups, as previously reported for in vivo activated splenic T cells (33). The analysis of other surface molecules confirmed (32) that most  $\gamma/\delta$  i-IELs are Thy-1<sup>-</sup> (see Table 1), while a higher proportion of  $\alpha/\beta$  lymphocytes are Thy-1<sup>+</sup> (not shown). It follows that fewer Thy-1<sup>+</sup> cells are detected in GF mice (Table 1).

Although considerably reduced, i-IEL  $\alpha/\beta$  cells in GF animals could contribute to the maintenance of  $\gamma/\delta$  T cells in the tissues (lymphokines, or other helper effects). We had previously observed that young adult, athymic (nude) mice contain reasonable numbers of i-IEL  $\gamma/\delta$  but almost no detectable  $\alpha/\beta$  cells. By studying i-IEL from GF nude mice, we could assess these influences. As shown in Fig. 4, by comparing euthymic SPF with GF animals, we confirmed the marked decrease of  $\alpha/\beta$  and the maintenance of  $\gamma/\delta$  cell numbers in GF animals. In nude animals, however, (in the absence of  $\alpha/\beta$  cells) the numbers of  $\gamma/\delta$  cells are threefold lower in GF as compared with SPF mice. That is, bacterial colonization does have an effect on i-IEL  $\gamma/\delta$  cell numbers, but this is only detectable in nude animals. Furthermore, by comparing SPF nude and normal mice, the complete thymic dependence of i-IEL  $\alpha/\beta$  is also shown, as well as the apparent thymic independence of  $\gamma/\delta$  cells. These results indicate that: (a) a fraction of  $\gamma/\delta$  i-IEL are independent of both bacterial colonization and  $\alpha/\beta$  cell activities; (b) in euthymic

animals, bacterial colonization has no effect on  $\gamma/\delta$  i-IEL numbers, indicating that self antigens and those contained in the food are enough to ensure their localization and pool size; and (c) in nude GF animals, bacterial colonization does influence i-IEL  $\gamma/\delta$  cells. This may be interpreted in two ways: only a fraction of i-IEL  $\gamma/\delta$  cells originate extrathymically and the repertoire of this subset is susceptible to local bac-



**Figure 4.** Numbers of  $\alpha/\beta$  and  $\gamma/\delta$  i-IEL T cells in euthymic and athymic, SPF and GF mice, as indicated. The data represent the mean of two independent mice analyzed in each group.

terial influences, in contrast with the majority of normal  $\gamma/\delta$  i-IEL that originate in the thymus and are independent of microbial antigens; alternatively, the local expansion of  $\gamma/\delta$  i-IEL is dependent on factors produced by  $\alpha/\beta$  cells, which are present though in reduced numbers in GF mice, and may be stimulated by food antigens. In any event, these results demonstrate  $\gamma/\delta$  "responses" that are completely independent of  $\alpha/\beta$  activities.

These present observations extend the long known heterogeneity of gut-associated T lymphocytes, which include cells with morphology, origins and traffic properties that distinguish them from most T cells in the periphery (34–37). Previous histological studies have demonstrated the selective localization of these lymphocytes among epithelial cells in the villi, although the T cell composition of i-IEL and lamina propria lymphocytes has not been found consistently different (38). Our observations, however, are at variance with a recent report (31), in which the representation of  $\alpha/\beta$  cells among i-IEL was not considered and therefore the effects of bacterial colonization in i-IEL composition and activation appeared to be due to  $\gamma/\delta$  T cell responses. There are two differences between that work and ours: first, we have directly enumerated  $\gamma/\delta$  and  $\alpha/\beta$  cells, while none of these class-specific antibodies were used in that study; second, we have analyzed the entire i-IEL population, by avoiding a density gradient centrifugation step, and eliminating contaminating epithelial cells from the analysis on the bases of their typical

high light scatter patterns and autofluorescence. The current belief that  $\gamma/\delta$  i-IELs display cytolytic activity (7, 31), particularly in response to bacterial colonization, is mainly based on those experiments. However, they provided no direct evidence for  $\gamma/\delta$  effector cells, since cytolytic activity was detected following enrichment of all CD8<sup>+</sup> cells in i-IEL preparations. Our results, therefore, question those conclusions by revealing the abundance of i-IEL CD8<sup>+</sup>  $\alpha/\beta$  cells, and by showing that such cells, but not  $\gamma/\delta$  lymphocytes, respond to bacterial flora.

As shown here, the antigenic load in the gut lumen is a major determinant of lymphocyte composition and activation in the complex microenvironment of the intestinal wall. Clearly,  $\alpha/\beta$  but not  $\gamma/\delta$  T cell localization to the intestinal wall is largely antigen-dependent. Although we have detected some variability in the total numbers of  $\gamma/\delta$  i-IELs in SPF mice originating from different colonies (unpublished observations), we could find no evidence for their localization and for local expansion being influenced by the intestinal flora in physiological conditions, that is, in the absence of infectious disease. These cells, therefore, seem to be essentially independent of bacterial stimulation. This behavior of i-IEL is similar to that of  $\gamma/\delta$  T cells homing to other epithelia. Thus, in fetal life, two other  $\gamma/\delta$  populations colonize skin and orogenital mucosa, independently of external antigens (5, 9, 12, 15).

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