STUDIES ON THE PERSISTENCE OF THE INDIVIDUAL STRAIN OF E. COLI IN THE INTESTINAL TRACTS OF THE INFANTS DURING THE PERIOD OF WEANING

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INTRODUCTION

The studies concerning diarrhoea of infants, viz., dispepsia, enteritis of infants, toxicosis, etc. have been the most serious problems among the pediatricians. The past studies on these problems ranged over wide fields and many brilliant results have been obtained. The physiological studies on the somatic factor, by which these diseases are caused, cleared the light on the movement of electrolytes, body fluid and acid basic composition in the cases of diarrhoea of infants, and contributed for the improvement of fluid infusion resulting in marked decrease of mortality from this disease. By the past studies on the climatic factor which has an important bearing as the cause of this disease, such as high and low temperatures and especially somatic adaptabilities, much knowledge has been accumulated on behavior of the autonomic nervous and endocrine systems, and then their relation to the habit of body. On the other hand from the fact that the significant effects are obtained with chemotherapeutic agents, such as sulfamide, streptomycin, chloromycetin, etc., roles played by intestinal flora as well as some kinds of viruses must be taken into consideration as causes of the disease. Then an attention was revived on Adam's view on the pathogenicity of coli bacteria. Since Kauffmann and his associates reported the antigenetical classification of coli bacteria, the study on this field became gradually active and lately the interest was concentrated on the pathogenicity of α -coli bacteria (O-111, B-4), β -coli bacteria (O-55, B-5), that both have similar antigenic construction: and that these coli bacteria are antigenetically the same with Adam's dyspepsy-coli bacteria A-4 and A-1^(1,2,3).

In order to have manifestation of pathogenicity of certain bacteria such as intestinal bacteria several conditions must be encountered after the organism invades into human intestine.

The first condition is that certain germs must invade the intestine and then proliferates and persists there for some period. The study of this idea has been

made extensively in the Department of Bacteriology, School of Medicine, Keio University with lower animals^(5, 6, 7, 8). But we have not heard any study concerning the persistence of individual strains in infants in order to analyse the cause of diarrhoea of infants. As the most fundamental experiment of infection and manifestation of infectious intestinal disease, we pursued the destiny of streptomycin—fixed coli bacteria named as “labelled” coli bacteria, which was given orally in the intestine of mice, and then my associates and myself tried the experiment on human body, and followed the proliferation of this organism in the intestines of newborn babies and artificially conditioned infants, and have reported about its effect on human body⁽⁹⁾. For last few years an antigenetic analysis of coli-form bacteria was reported by Scandinavian scientists such as Kauffmann, Vahlne and Knipschild etc., and thus the base of classification of bacteria was established, which had been regarded as the most difficult problems⁽¹⁰⁾.

This report bears on the observations on the persistence of coli bacteria in the intestinal tracts of the infants in their weaning period in natural and healthy state.

METHODS OF STUDIES

A. Course of Observation

Stools of three healthy infants were examined every few days. The strains of coli bacteria which have appeared frequently and larger in number during the first one month were considered persisting coli bacteria in their intestinal tracts. With anti-O-serum of rabbit prepared with these strains, the coli bacteria, which shows O-agglutination to these antiserum, were considered as the same strains antigenetically. Repeating this procedure for a certain period of time we were able to determine the period of time for which the strains persisted in the intestinal tracts of the infants.

B. Subjects and Durations of Observation

Observations were made on three healthy infants; namely the first case three-month-old infant of breast feeding, the second case two-month-old infant of partial feeding and the third case four-month-old infant of artificial feeding, designated hereafter respectively ■■■, ■■■, and ■■■. The durations of observation were seven to eleven months beginning September 1950.

C. Determination of Individual Strains of Coli Bacteria

Culturing stool of every infant on Endo plate every few days for a month

and its result was observed. Considering the strains forming colony of same character as the same genus temporarily, certain strains which appeared predominantly in every occasion were fished and examined its biological character. (Table 1, 2 and 3) Excluding the strains belonging to the intermediate type and to aerogenes type according to Parr's classification, the strains of coli type character having the same biological character and the same colonial appearance are considered as one genus, and selecting the strains that appeared most frequently and predominantly rabbits were immunized to the antigen thereof.

D. Preparation of Anti-O-Serum to Individual Strains of Coli Bacteria

Heat-killed (2 hours at 100°C) saline suspension of 18 hours culture of the strains of E. coli selected as described above, containing 1 mg per ml of 0.25% formalin was inoculated intravenously to rabbits in four divided doses of

Table 1
Data on the biological character of 8 strains of coliform bacteria isolated from subject I, W over a period of the first one month.

Date of collection of specimens	19/9	27/9	3/10	5/10	10/10	14/10	16/10	
Strain for preparation of O-sera	○		○			○		
Strain collected	W-1	W-5	W-7	W-9	W-10	W-11	W-12	W-14
Motility	—	—	+	+	+	+	+	—
Indol formation	+	+	+	+	+	+	+	+
Methyl red reaction	+	+	+	+	+	+	+	+
Voges-Proskauer reaction	—	—	—	—	—	—	—	—
Sodium citrate test	—	—	—	—	—	—	—	—
Lactose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Glucose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Sucrose	—	—	+	+	+	+	+	—
Salicin	+	+	—	—	+	+	+	+
Degree of appearence	≡	≡	≡	≡	+	≡	≡	≡
Type	C	C	C	C	C	C	C	C

⊕: formation acid and gas

+: acid formation without gas

Type C: coli type

I: intermediate type

A: aerogenes type

Table 2
*Data on the biological character of 19 strains of coliformbacteria isolated from subject I. H.
 over a period of the first one month.*

Date of collection of specimens	18/9	23/9	25/9	28/9	2/10	7/10	10/10	14/10	16/10
Strain for prepara- tion of O-sera			○	○			○		
Strain collected	H-1	H-4	H-5	H-6 H-7 H-8	H-9 H-10 H-11	H-12 H-13 H-14	H-15 H-16 H-17	H-19 H-20	H-22 H-24
Motility	-	+	+	+	+	+	+	+	+
Indol formation	-	+	+	+	+	+	+	+	+
Methyl-red reaction	-	+	+	+	+	+	+	+	+
Voges-Proskauer reaction	-	-	-	-	-	-	-	-	-
Sodium citrate test	+	+	-	-	-	-	+	-	-
Lactose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Glucose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Sucrose	⊕	-	-	-	-	-	⊕	-	-
Salicin	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Degree of appearance	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Type	A	I	C	C	C	C	C	A	A

Table 3
Data on the biological character of 17 strains of coliformbacteria isolated from subject I. G over a period of the first one month.

Date of collection of specimens	18/9	27/9	30/9	4/10	7/10	10/10	14/10	16/10
Strain collected	G-1 G-2 G-3	G-4 G-5 G-6	G-7	G-8 G-9	G-10 G-11	G-14	G-15 G-16 G-17	G-18 G-19
Motility	- - -	+ - -	+	+	-	-	+	+
Indol formation	+	+	+	+	+	+	+	+
Methyl-red reaction	+	+	+	+	+	+	+	+
Voges-Proskauer reaction	- ±	- -	-	-	-	-	-	-
Sodium citrate test	- ±	- -	-	-	-	-	-	-
Lactose	⊕ ⊕ ⊕	⊕ ⊕ ⊕	⊕	⊕ ⊕ ⊕	⊕ ⊕ ⊕	⊕	⊕ ⊕ ⊕	⊕ ⊕ ⊕
Glucose	⊕ ⊕ ⊕	⊕ ⊕ ⊕	⊕	⊕ ⊕ ⊕	⊕ ⊕ ⊕	⊕	⊕ ⊕ ⊕	⊕ ⊕ ⊕
Sucrose	- - -	- + -	-	-	-	-	+	+
Salicin	- - -	+	+	+	-	+	-	-
Degree of appearance	⊕ + +	⊕ + ±	⊕	⊕	⊕	+	+	+
Strain for preparation of O-sera	○	○ ○						
Type	C C? C	C C C	C	C C	C C	C	C C C	C C C

0.3 c.c., 0.5 c.c., 1.0 c.c., and 2.0 c.c.

The rabbits thus immunized had anti-O-sera having agglutination titers of 800:1 to 3200:1.

E. Isolation and Examination of Coli Bacteria

Samples of stools of each infant were examined as frequently and as long period of time as possible. Each sample was plated out on Endo media and fished out 20 colonies of coliform bacteria. These were planted on slant agar media and kept as test strains for further examinations. The organisms from each test strain were cultured in Bouillon for 18 to 20 hours and then killed by heating at 100°C for 1 to 1½ hours.

Using this suspension as antigen agglutination tests were made at first with mixtures of anti-O-sera prepared with *E. coli* strains isolated from the same individual infant. One c.c. of a mixed anti-O-sera having titers of 200:1 was placed in a test tube and then 2 drops of the antigen was added. Each tube was kept at 37°C for 2 hours and read after 24 hours in room temperature. When this test gave positive reaction, the types of each strain was determined by quantitative agglutination and absorption test. Further tests were greatly facilitated by the kindness of Dr. H. Hiroki of the Kitasato Institute, who gave us the standard anti-O-sera prepared according to Kauffmann's type classification of *E. coli*.

F. Other Examinations

While we were persuing the persistences of coli bacilli we also kept watch on the other intestinal flora such as Gram positive as well as negative organisms. At the same time careful record was kept on details of feedings, nature of stool and health condition of every infant and from these records we attempted to find out some relations of the intestinal flora and the condition of the infants.

RESULTS OF THE STUDIES

I. The results of our observations on the case [REDACTED] are shown in Table 4.

This test was started, when the infant was three months old, and had been continuously fed on breast since birth until be weaned at the age of six months. The result of antigenetic analysis on the isolated coliform bacteria is shown in Table 5.

The observation was continued for 329 days, and 570 test strains were isolated and examined. H-5 strains isolated in this case persisted predominantly throughout the period of our observation. H-8 strains also persisted predominantly.

Table 4
Results obtained from subject [REDACTED]

Date of collection of stool specimen	Number of cultures in specified O group										B. proteus	Character of stool	Gram-stained preparates			Diet and pathological symptoms of infant
	H-5 (O-2)	H-8	H-17	O-3	O-21	O-9	O-6	O-11	O-20	Un-grouped			Gram positive cocci	Gram positive bacilli	Gram negative bacilli	
23/9/50'	+										+	L.M.G.				Breast milk only.
25/9	+										+	L.M.G.				Common cold, Fever (—)
28/9	+	+									+	N.				diarrhea 4 times a day
2/10	+										+	L.M.				
7/10	+										+	N.M.				
10/10			+								+	N.				
14/10	+										+	N.				
16/10	+	+									+	N.				
21/10	6	2								12	+	N.	+	##	+	
25/10	17									3	+	N.	+	##	±	
2/11	18	1								1	+	N.	+	##	±	
10/11	6	6								8	+	L.M.	##	##	±	
15/11	18									2	—	N.	+	##	±	
20/11	17									3	—	N.	+	##	+	
30/11		20								0	+	L.M.G.	##	##	+	Since yesterday diarrhea
2/12	19									1	+	N.	+	##	±	
13/12	16	2								2	+	N.M.	+	##	+	
17/1/51'	11	4								5	—	N.	+	##	±	Start to wean
23/1										20	+	L.	+	##	+	
6/2	19									1	+	L.	+	##	##	
20/2		11	3							6	+	W.	+	##	+	Diarrhea 5 times a day
1/3	18									2	+	N.	+	##	+	
20/3				7						13	+	L.	+	##	+	
24/3	1			15	4					0	+	N.	##	##	+	
27/3	4			3	10					3	+	L.	+	##	+	
6/4	18				2					0	+	N.	+	##	##	
11/4				3	7			10		0	+	L.	+	##	+	
16/4	20									0	+	L.M.G.	+	##	+	Today diarrhea
18/4	10							10		0	+	W.M.G.	+	##	+	Diarrhea
24/4					4					16	—	L.	+	##	##	
4/5	7	2								11	—	N.	—	±	##	
9/5						8	4			8	—	L.M.	+	+	##	
15/6	6							12		2	+	N.	+	±	##	
27/6				2	4			14		0	+	W.M.	##	##	##	Since 2 days diarrhea
5/7	4	10							6	0	+	L.	—	##	##	
9/8	13	3			1					3	+	N.	—	+	##	

Character of stool—N: normal, L: loosed, W: watery, M: mucous, G: granulated

Table 5
Data on 570 cultures of *E. coli* isolated from subject [REDACTED]
over a period of 47 weeks.

Age in month	Period of collection of specimens	Days in period	Number of specimens	Number of cultures	Number of cultures in specified O group										
					H-5 (O-2)	H-8	H-17	O-3	O-21	O-9	O-6	O-11	O-20	un- grouped	
3~4	23/9~16/10/50'	24	8	10	7	2	1								0
4	17/10~25/10	9	2	40	23	2									15
4~5	26/10~30/11	36	5	100	59	27									14
5~6	1/12~13/12	13	2	40	35	2									3
6~7	14/12~23/1/51'	41	2	40	11	4									25
7~8	24/1~20/2	28	2	40	19	11	3								7
8~9	21/2~27/3	35	4	80	23			25	14						18
9~10	28/3~24/4	27	5	100	48			3	13			20			16
10~11	25/4~10/5	16	2	40	7	2				8	4				19
11~12	11/5~27/6	47	2	40	6			2	4			26			2
12~13	28/6~6/9	9	1	20	4	10							6		0
13~14	7/7~9/8	34	1	20	13	3			1						3
	Total	329	36	570	255	63	4	30	32	8	4	46	6		122

During the first 151 days both H-5 and H-8 strains were predominant in number and the latter gave its place to O-3 and O-21 strains for the next two months. However they were soon replaced by H-8 strains again.

At any rate both H-5 and H-8 were predominant most of the times having found 318 strains out of 570, or 57.6% of all the isolated strains. Thus it can be said that these two were resident strains, and all the others were only transient. Diarrhoea occurred 5 times in the whole period of the observation without any other clinical symptoms. Twice out of five there were O-11 strain found in his stools, but it might have been only by chance. There hardly was any correlation between diarrhoea and its resident strains, because the frequency of the appearance of H-5 strains was not changed even at the time of diarrhoea. However, a significant fact is that [REDACTED] suffered from diarrhoea just before and after the transition from H-8, to O-3 and to O-21 and of the disappearance of these latter two.

II. Table 6 is the results of case [REDACTED]

This was a case of infant two-month-old, fed on breast for the first two months after birth. At the age of 3 months due to insufficiency of mother's milk a mixed feeding was instituted and continued 1½ months and then changed entirely to the artificial feeding and at 6 month old semi-solid food was added

Table 6
Result obtained from subject [REDACTED]

Date of collection of stool specimens	Number of cultures in specified O group					B. proteus	Character of stool	Gram-stained prepares			Diet and pothological symptoms of infant
	W-1 (O-11)	W-7 (O-8)	W-12 (O-3)	O-2	Un-grouped			Gram positive cocci	Gram positive bacilli	Gram negative bacilli	
19/9/50	+					—	N.				breast feeding
27/9	+					+	L.M.				
3/10		+				—	N.				
5/10	+	+				—	N.				
10/10	+	+	+			—	L.M.				
14/10	+		+			+	N.				
16/10	+		+			—	N.				
23/10	11	6		2	1	—	N.	—	##	+	
30/10	15			5	0	+	N.	##	##	##	
10/11	17				3	—	N.	+	##	##	
15/11	3			10	7	+	N.	+	##	##	+ powdered milk + cow milk
18/11	19				1	+	N.	+	##	##	
25/11	15			4	1	—	N.	—	+	##	
30/11	20				0	+	L.	##	+	##	
17/12	7	2		2	9	+	N.	##	+	##	
23/1	8			12	0	+	N.	+	+	##	
30/1	19				1	+	N.	+	+	##	
6/2	14			3	3	—	W.M.G.	##	+	##	
1/3	16			4	0	—	N.	+	+	##	
15/3				18	2	+	N.	##	+	##	
16/3	15			5	0	—	L.	+	+	##	loosed stool Ps. aeruginosa (+), no symptoms
27/3			2		18	+	L.	+	##	+	
6/4		8	10	2	0	—	N.	##	±	±	Eruption (+) Fever 39.3°C cough (+) cow milk only improved, good appetite
11/4	8			10	2	+	N.	+	±	##	
20/4	17				3	+	N.	##	±	##	

Table 7
Data on 372 cultures of *E. coli* isolated from subject [REDACTED]
over a period of 31 weeks.

Age in month	Period of collection of specimens	Days in period	Number of specimens	Number of cultures	Number of cultures in specified O group				
					W-1 (O-11)	W-7 (O-8)	W-12 (O-3)	O-2	Un-grouped
2~3	19/9~22/10/50'	34	7	12	6	3	3		0
3	23/10~30/10	8	2	40	26	6		7	1
3~4	31/10~30/11	31	5	100	74			14	12
4~5	1/2~17/12	17	1	20	7	2		2	9
5~6	18/12~30/1/51'	44	2	40	27			12	1
6~7	31/1~6/2	7	1	20	14			3	3
7~8	7/2~27/3	49	4	80	31		2	27	20
8~9	28/3~20/4	24	3	60	25	8	10	15	2
Total		214	25	372	210	19	15	80	48

for weaning. Table 7 shows the results of coli bacteria isolated from this case. Observation of this case continued for 214 days, from the second to ninth month of his birth, and 372 strains were isolated. W-1 strains, coinciding with Kuffmanns O-11, isolated from stools of this case was predominant throughout our observation, and one strain corresponding to Kauffmann's O-2 strain was predominant for six months from the beginning of the mixed feeding.

The resident strains in this case were W-1 and O-2, which were predominant throughout the period of our observation having found 290 times out of 370, or 77.9% of all the test strains. W-7 and W-12 appeared few times but they were only transient and did not persist any length of time, in the other words almost all of bacterial flora in the stools of this case were W-1 and W-2. At the age of seven months the child had diarrhoea without any other clinical signs, and *Ps. aeruginosa* was found but the persistence of the resident strains was not altered by it. The child had measles when eight months old and was treated with penicillin and sulfadiazine to prevent possible complication. During this treatment there was no changes in the persistences of the resident strains, except a temporal disappearance of W-1 strain for a short period of time.

III. The results of the case [REDACTED] are summed up in Table 8.

The child was four months old, when our observation begun, fed on pottage and bouillon soup, and bread and biscuit were added from the fifth month on. The observation was continued for 268 days from the fourth to twelfth month, and 276 strains were isolated. G-1 and G-3 strains persisted predominantly for

the first two months as the table shown. On the other hand, O-12 strain began to appear one and a half months later, gradually replacing G-3 strain and increased in the number and finally it expelled G-1 as well and persisted predominantly for the next three months. But it was again replaced by O-21 and O-5 strains. Throughout the observations on this case only one or two strains proliferated predominantly, but the period of their persistence was relatively short, and suggested that antigenetically different species of strains competed each other for persistence. It appears from the results that there is some suggestive relation between breast feeding and replacement of O-3 by O-12 and glue feeding with replacement of O-12 by O-21 or by O-5. It was impossible to have any conclusive evidence, however, to draw any conclusion on these points, because the examinations had been made at too long intervals.

IV. From the examinations of Gram-stained specimens of stools, we were not able to make any conclusion on the relation between replacement of bacterial flora and diarrhoea in these cases.

DISCUSSION

1. Various organisms have been considered as causative agents of diarrhoea in infant. Especially coli bacteria was taken up for study to clarify the mechanism of the infection and diarrhoea with special reference to their antagonistic action to *B. bifidus* and *B. acidophilus* in intestine and in test tube.

Recently the antagonistic actions of coli bacteria against *Shigella dysenteriae* and of one strain against another strain of coli bacteria attracted our attention for manifestation of diarrhoea in infant. We pursued the fates of "labelled" coli bacteria given orally. We found out that the administered strains did not persist in the intestines of the infants as well as in experimental animals such as mice, and usually they disappeared in a few days⁽⁹⁾. If, however, the infant or experimental animals are subjected to certain conditions, which disturb the intestinal flora, the persistence of the test strains are much prolonged. This result is in accord with the results obtained by Hirata in his experiments with mice⁽¹⁴⁾. These facts indicate that there is a certain equilibrium among the intestinal flora and individually there are a few strains which are fixed and persisted in the intestines and resist against proliferation of freshly introduced coli bacteria.

The results of our observations indicate that antigenically one to three species of strains had been fixed in the intestine and multiplied for certain period. Even

at the weaning period there were a few resident strains which persisted for a certain period of time. These results were in many points in accord with the results obtained by Horie in his study of biological character of coli bacteria in adult, and also that made by Nishimoto, who studied in infant for long period of time by means of anti-O-H-serum^(15,16). But their conclusions have been made rather on meagre bases antigenetically. Further more our results are in accord, particularly in reference to resident strains and persistence of *B. coli* in adults which had been observed by Kauffmann, Stuart and Sears⁽¹⁷⁾.

2. We found that there are two varieties of strains, one resident strains which multiply vigorously and persist for certain periods of time and the other, transient strains which may multiply temporarily but do not persist. But these properties are not absolute. Even the resident strains may be replaced by transient strains, as seen in the case [REDACTED] if some derangements have been created in the intestines.

As seen in the H-8 strain in the case [REDACTED] there are some resident strains where the property of persistency is unstable. While O-3 and O-21 strains, which are transient strains, are multiplying vigorously, H-8 remained quiescent. It appears that the growth of a resident strain may be inhibited when a certain condition in the intestine is altered. This view is upheld by the results, as reported in our previous paper of our observations on human subjects with the "labelled" coli bacteria.

This phenomenon in part is due to the antagonistic actions of various strains of coli bacteria in intestines and partly due to the changes of food as seen in the case of [REDACTED] and [REDACTED].

3. [REDACTED] suffered from mild diarrhoea for a day without any clinical symptom during our observation. *Ps. aeruginosa* was then isolated but it soon disappeared without exerting any influence on the persistence of the resident strains, W-1 and W-2. This is an interesting fact to clarify the role of resident strain in the intestine.

4. According to the reports of Stuart and Sears, etc., they observed that, though not invariably the adults suffered from diarrhoea at the time of transition of the resident coli bacteria. We also observed that diarrhoea occurred at the time of disorder and transition of the resident coli bacteria in the cases of [REDACTED] and [REDACTED]. As we have reported in our previous paper on our observations with the "labelled" coli bacteria in human body, we found that disorder and transition of the resident coli bacteria alone did not cause any clinical symptom. From this fact it may be said that the disorder and transition of the organism alone do not cause diarrhoea. As for coli bacteria itself, the relation between resident strain

and diarrhoea is not only a problem of equilibrium among the strains, but also it is a problem of importance to study the relationship between the pathogenity and antigenetic structure of coli bacteria.

CONCLUSION

1. The transition of coli bacteria in intestine was observed by means of O-antigenetic analysis on three healthy infants during weaning period for period of time at before and after weaning period.

2. It is observed that there are one to three strains of resident coli bacteria, which persist and multiply for certain period, and also transient coli bacteria, which appear temporarily and disappear.

3. The predominance of the resident strains is not absolute but it may be replaced by the others, when some conditions are exerted on the intestine.

4. The circumstance which causes such phenomenon is still not clear, but it seems to us that there is some relation with feeding of infant. In the infant of artificial feeding the transition of the resident strains is more frequent than in the others.

5. Some times when diarrhoea occurs the transition of the resident strain was observed, but their relation remains unexplained.

Table 9
*Data on 276 cultures of E. coli isolated from
subject [redacted] over a period of 38 weeks.*

Age in month	Period of collection of specimens	Days in period	Number of specimens	Number of cultures	Number of cultures in specified O group									
					G-1	G-3 (O-11)	G-4	O-12	O-21	O-5	G-5 (O-3)	O-23	O-7	Un- grouped
4~5	18/9~16/10/50'	29	8	16	6	6	3				1			0
5	17/10~27/10	42	2	40	34	1								5
5~6	28/10~20/11	23	4	80	39	4	1	12						24
6~7	21/11~12/12	22	1	20				10						10
7~8	13/12~15/1/51'	32	1	20				16						4
8~9	16/1~20/2	36	1	20				8	1		6	2		3
9~10	21/2~24/3	32	1	20					4	14				2
10~11	25/3~6/4	23	1	20					7	13				0
11	17/4~1/5	15	1	20	20									0
11~12	2/5~15/5	14	1	20									3	17
Total		268	21	276	79	31	4	46	12	27	7	2	3	65

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