Table 1. Chromosome types in three groups of *A. stephensi* adult females with different times of emergence.

Group	Mosquitoes examined (No.)	Frequencies of:					
		ST/ST		ST/KR		KR/KR	
		No.	%	No.	%	No.	%
1	143	98	68.53	42	29.37	3	2.10
2	146	12	8.22	128	87.67	6	4.11
3	139	10	7.19	56	40.29	73	52.52
Control	142	36	25.35	75	52.82	31	21.83

not readable. The results are shown in Fig. 1 and Table 1. Differences in the frequencies of ST/ST, KR/KR, and ST/KR karyotypes were found among the three groups, all of which showed a chromosomal constitution significantly different from that in the control group ($P < .001$). The alternative gene arrangements showed clinal frequency changes from group 1 to 3, indicating a close relation between type of chromosome and the pattern of adult emergence. The mosquitoes emerging first were mostly homozygous ST/ST; the heterozygotes, ST/KR, reached the highest frequency during the middle part of the emergence period; and the homozygotes KR/KR were mainly recorded among those mosquitoes emerging last. In other words, each of the three karyotypes showed a distinct emergence peak, the heterokaryotype being intermediate between the two homokaryotypes.

Because of differences in the period of larval development in the three inversion genotypes, the genotype frequency differed significantly in mosquitoes that emerged on consecutive days. This accounts, in part, for the changes in the shape of the emergence curves. However, the reported relations between the emergence periods of the three inversion karyotypes were recorded on the three principal, consecutive days of emergence in a total of seven replicates of the experiment just described, four of them with the Iraqi strain and three with the Irani strain. Similar results were also obtained in two experiments with the time of pupation. Other experiments indicate the following:

1) The selection of the Iraqi strain for earlier adult emergence resulted in a rapid and highly significant increase in the frequency of ST. Parallel selection for delayed adult emergence resulted in a significant increase of KR.

2) The mean emergence time was different in homozygous ST and KR stains extracted at random (without selection depending on emergence time) from the Iraqi strain. The ST strain showed an early emergence pattern, while the KR strain showed a delayed emergence pattern that also appeared relatively less variable.

3) The differences reported in 2) were not found in the strains from India and Pakistan that were monomorphic for the ST and KR gene arrangements, respectively. The emergence curves of these strains resemble those of the polymorphic strains.

4) The intermediate emergence pattern of the heterokaryotype, typical in the polymorphic strains, was not observed in an F_2 sample obtained from a cross between the KR Pakistani strain and the ST Indian strain.

The evidence indicates that the difference in the emergence time between the carriers of the ST and KR gene arrangements is not determined by the inversion in itself, but is a result of a process of heteroselection, the two homokaryotypes being coadapted in the sense used by Dobzhansky (*7*). The Indian strain is monomorphic for ST and the Pakistan one is monomorphic for KR, whereas the strains from Iraq and Iran are polymorphic. It is likely that, in the polymorphic populations, the chromosomes with the two gene arrangements carry different gene complexes that are coadapted, and that interact favorably in heterozygous carriers. This situation produces a balanced polymorphism and appears to originate the difference in emergence time reported here. If this hypothesis is correct, the process of inversion coadaptation would constitute a genetic device for the production of diversified genetic regulatory systems. Such polygenic variants, determining diverse developmental (and behavioral) pathways, are likely to play a role in colonization of ecologically marginal zones and in the occupation of new ecological niches. This might suggest a causal relation between chromosomal rearrangements and speciation (*2, 8, 9*).

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Restoration of Antibody-Forming Capacity in Cultures of Nonadherent Spleen Cells by Mercaptoethanol

Abstract. *Populations of mouse spleen cells, without those cells that adhere to glass or plastic, exhibit little or no capacity for formation of antibody to sheep erythrocytes in the Mishell-Dutton culture system. When 5×10^{-5}M mercaptoethanol is added to the culture medium, the antibody-forming capacity of these nonadherent spleen cells is restored to that of unfractionated spleen cells.*

Dissociated spleen cells from normal inbred mice can be separated into two different populations on the basis of their adherence to plastic or glass. Both the adherent and the nonadherent cell populations are required for optimal immune response in vitro to sheep erythrocytes (*1*). The adherent cell population (i) is rich in phagocytes but does not contain the precursors of antibody-forming cells (*2*); (ii) has a function that is radioresistant (*3*); and (iii) can be replaced by appropriate numbers of peritoneal macrophages (*4–6*) or, in some instances, by supernatants of macrophage cultures (*6, 7*). We show here that addition of mercaptoethanol, in low concentrations, to cultures of nonadherent cells restores their capacity to form antibodies in

vitro. The use of mercaptoethanol in the Mishell-Dutton culture system was first suggested by Click (*8*).

Spleen cells were obtained from 3- to 6-month-old DBA/2J mice. The cells were cultured according to the method of Mishell and Dutton (*9*). Nucleated spleen cells were always used at a concentration of 1×10^7 cells per culture, and sheep erythrocytes as antigen were added at 5×10^6 cells per culture. The sheep cells were present for the entire 5-day incubation period. Nonadherent cells were prepared by the method of Mosier (*1*) with some modification; 4×10^7 nucleated cells in 3 ml medium (supplemented Eagle's minimal essential medium containing 10 percent fetal calf serum) were placed in a 60 by 15 mm plastic tissue culture dish (Falcon) and incubated without agitation at 37°C in a gas mixture of 10 percent CO_2, 7 percent O_2, and 83 percent N_2 for 2 hours. After gentle mixing and careful aspiration, the nonadherent cells were transferred to another dish and incubated again under the same conditions for an additional hour. The nonadherent cells were then collected, washed, and suspended in fresh medium to give a concentration of 1×10^7 nucleated cells per culture. The number of antibody-forming cells was determined on day 5 by the Jerne direct hemolytic plaque assay method (*10*).

The nonadherent cell population prepared in this manner, and cultured with sheep red blood cells in the Mishell-Dutton system, developed very few, if any, plaque-forming cells. The plaque-forming ability of these nonadherent cells was restored, totally or in part, by addition to the culture system of adherent spleen cells, or of peritoneal macrophages in appropriate numbers, as reported by others (*1, 5, 6*). Addition of mercaptoethanol (1 to $10 \times 10^{-5}M$) to the medium containing the nonadherent spleen cells and sheep red cells, at the initiation of the culture, resulted in restoration of the plaque-forming response to that of the spleen cell population containing both adherent and nonadherent cells (Table 1). Addition of mercaptoethanol to unseparated spleen cell cultures also resulted in generally higher and more consistent numbers of plaque-forming cells. At a concentration of $1 \times 10^{-6}M$, mercaptoethanol in the medium produced a less than optimal number of plaque-forming cells; mercaptoethanol at $1 \times 10^{-3}M$ was toxic to the cells.

The mechanism of action of mercaptoethanol in this system is not clear.

Table 1. Effect of mercaptoethanol on the in vitro immune response of mouse spleen cells to sheep red blood cells.

Mercaptoethanol ($5 \times 10^{-5}M$)	Plaque-forming cells per culture* on day 5 in experiments:			
	1	2	3	4
	Unseparated cells			
−	475	1760	645	2250
+	1250	1800	1500	2520
	Nonadherent cells			
−	10	10	55	80
+	635	365	1690	2500

* Start of culture, 1×10^7 nucleated cells.

The nonadherent spleen cell population [prepared by our method or by other procedures (*6*)] is not likely to be depleted entirely of macrophages or of macrophage precursors, but those remaining are present in numbers too small to facilitate antibody production. After 1 day of stationary culture of the nonadherent cells, scattered mature macrophages (total number, 3×10^3, or less, per culture) were seen adhering to the dishes. This is in contrast to the unseparated culture of spleen cells that contained large numbers (about 1×10^5) of macrophages per dish at the same time. After 5 days of culture of the nonadherent spleen cells, approximately 1×10^4 macrophages were present in each dish, the number and appearance of these macrophages being the same whether or not mercaptoethanol had been added. These macrophages which appeared during culture of nonadherent spleen cells were probably derived for the most part by maturation of macrophage precursors in the spleen. Since macrophages are only required during the first 24 hours of culture (*11*), the late-appearing macrophages may not be important to the immune response. During the period of culture there was no evidence that mercaptoethanol stimulated proliferation of the residual small mononuclear phagocyte population. Mercaptoethanol might have potentiated the action of the small number of residual macrophages, a possibility that cannot be ruled out at present.

When sheep red cells were incubated with mercaptoethanol, and were then washed, and were added to cultures of nonadherent spleen cells, there was no restoration of antibody-forming capacity. These sheep red cells treated with mercaptoethanol retained their antigenic capacity, however, in cultures containing both adherent and nonadherent spleen cells. Incubation of sheep red cells for 3 hours in the Mishell-Dutton culture medium, with or without added mercaptoethanol, resulted in release into the medium of antigens or of fragments of red cell membrane that do not sediment at 1000*g* for 15 minutes. The amount of such "soluble" red cell material released was sufficient to stimulate significant plaque-forming response upon addition of unseparated spleen cells and upon continued culture. Nonadherent spleen cells formed plagues when mixed with these sheep red cell supernatants only when mercaptoethanol was also present, either as a result of its transfer to the culture system (in the case of the supernatant of sheep cell obtained after preliminary incubation in the presence of mercaptoethanol), or as a result of its addition (in the case of the sheep red cell supernatant obtained without prior incubation with mercaptoethanol).

Mercaptoethanol may act either on the medium or on the spleen lymphoid cells directly to enhance the immune response. We found that the nonadherent cell cultures had low viability and very few transformed cells after culture for 5 days (*5*), whereas cultures of the unseparated spleen cells or of nonadherent cells with added mercaptoethanol showed high viability and up to 40 percent transformed cells, whether or not sheep red cells were included in the culture.

The results raise the possibility that some of the functions of adherent cells or macrophages in the Mishell-Dutton culture system may be replaced by such simple substances as mercaptoethanol. The cells may release into the medium, or may transfer directly to lymphoid spleen cells, some reducing substance essential for the activation of lymphocytes.

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