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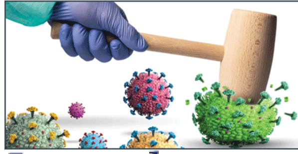

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LABELING CHARACTERISTICS AND SEPARATION OF Ia ANTIGEN SUBUNITS[1]

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Experiments with biosynthetic incorporation of ^{3}H-amino acids into murine and guinea pig Ia antigens have indicated that these antigens consist of two polypeptide chains of 33,000 and 25,000 daltons, respectively, occasionally linked by disulfide bonds into a 58,000 dalton molecule. In contrast, studies with lactoperoxidase-catalyzed radioiodination have indicated that these Ia antigens consist of only a single chain of 25,000 daltons. We therefore undertook a study to explore the basis of these discrepant results. Since ^{3}H-tyrosine labeled both chains well, the lack of tyrosine residues in the 33,000 dalton chain could not be the explanation for the lack of radioiodination. However, by partially purifying the Ia antigen preparation with *Lens culinaris* (lentil) lectin affinity chromatography before immunoprecipitation and by increasing the resolution of analysis by using discontinuous-SDS polyacrylamide gel electrophoresis, it was possible to show that the 33,000 dalton chain was in fact radioiodinated, though still poorly so relative to the 25,000 dalton chain, and that a radioiodinated 58,000 dalton molecule could be detected. These experiments suggest that the 25,000 dalton chain is more exposed to the external cellular environment, and thus more readily iodinated by lactoperoxidase. In addition, the studies indicate that the choice of labeling method, purification procedures, and analytical methods must be taken into account when interpreting experimental results.

Ia antigens are surface glycoproteins which are genetically determined by the major histocompatibility complex (MHC)[4] of mice, guinea pigs, and other species. They are of particular interest because they are determined by genes that are closely linked or identical to the immune response (Ir) genes, and appear to be directly involved in the control of the immune response (1).

The Ia antigen molecules have been analyzed by solubilizing radiolabeled lymphoid cells with nonionic detergents, binding the antigens to alloantibody, and precipitating the immune complexes with an anti-globulin reagent or with protein A-bearing staphylococci (2–8). The solubilized complexes have been examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE).

Two radiolabeling methods have been widely used by workers in the area. First, biosynthetic labeling by short-term culture of cells with a radiolabeled amino acid or monosaccharide precursor (2, 3, 7), and second, surface labeling by the lactoperoxidase-catalyzed radioiodination technique (3–6). Electrophoretic analyses of Ia antigens labeled by these two methods have led to some disagreement on the number of Ia polypeptide chains present in the mouse and guinea pig, and on the nature of the association of the polypeptide chains in the guinea pig (5, 7). We have compared these methods in both species and have been able to resolve the apparently discrepant results.

MATERIALS AND METHODS

Animals. B10.A and DBA/2 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Strain 2 and Strain 13 guinea pigs were obtained from the Division of Research Services, National Institutes, Bethesda, Md.

Alloantisera. Mouse alloantisera were the generous gift of Dr. C. S. David. Guinea pig alloantisera were kindly provided by Dr. E. M. Shevach. Table I lists the combination of animals in which the sera were raised, and the determinants detected by each antiserum.

Radiolabeling. 1) Biosynthetic labeling. Mouse spleen cells or guinea pig lymph node cells were incubated at 5×10^7/ml in serum-free minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 200 μCi/ml ^{3}H-leucine (80 Ci/mM New England Nuclear Corp., Boston, Mass.) as the sole leucine source for 5 hr at 37°C in a humidified atmosphere containing 5% CO_2. Labeling with ^{3}H-tyrosine was similar, except that the medium contained 100 to 200 μCi/ml of ^{3}H-tyrosine (50 Ci/mM, New England Nuclear) as the sole tyrosine source. 2) Lactoperoxidase-catalyzed surface iodination. Cells were suspended at 10^8/ml in phosphate-buffered saline, pH 7.4, to which 25 μg/ml of lactoperoxidase and $Na^{125}I$ (Amersham-Searle, Arlington Heights, Ill.) at 1 mCi/ml were added. Hydrogen peroxide was added to a final concentration of 8 μM, and this last addition was repeated twice at 5-min intervals, for at total reaction time of 15 min.

Solubilization and purification of Ia antigens. Labeled cells were solubilized by 0.5% NP40 in 0.01 M Tris, 0.15 M NaCl, pH 7.4, and the insoluble material was removed by ultracentrifugation at 100,000 × G for 60 min. The Ia antigens were partially purified by lentil lectin affinity column chromatography (9), isolated by immunoprecipitation, and analyzed by SDS-PAGE (8).

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[4] Abbreviations used in this paper: SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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TABLE I

Alloantisera

Species	Recipient	Donor	Target Cells	Determinants Detected[a]
Mouse	B10.A	BALB/c (Meth A fibrosarcoma)	DBA/2	H-2.31 (H-2K^d)
	(B10 × A) F_1	B10.D2	DBA/2	Ia.11,16 (I-A^d) also some H-2.31
	A.TH	A.TL	DBA/2	Ia.7 (I-C^d)
	(B10 × HTI) F_1	B10.A (5R)	B10.A	Ia.7 (I-C^d)
Guinea pig	Strain 13	Strain 2	Strain 2	Ia.2,4
	Strain 2	Strain BE	Strain 13	Ia.1,7

[a] Lists only those determinants which were best detected in the particular antiserum/target combination.

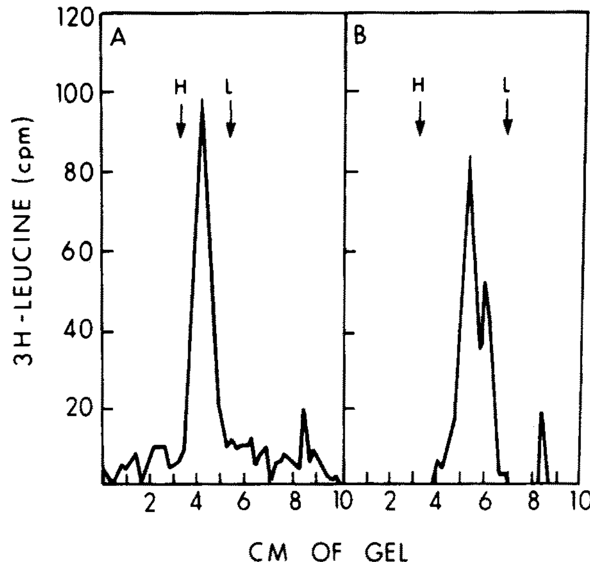

Figure 1. Comparison of SDS-PAGE buffer systems. ^{3}H-leucine-labeled Ia molecules from B10.A mouse cells were precipitated with the antiserum (B10 × HTI) F_1-anti B10.A (5R). The precipitates were solubilized with the buffers appropriate for each system, mixed with reduced and alkylated ^{14}C-labeled IgG (MPC-11 myeloma line), and electrophoresed. *A*, analysis with a nonstacking buffer system (11); only a single Ia component of 34,000 daltons can be discerned. *B*, analysis with a stacking buffer system (8). Two components of the Ia molecule of 35,000 daltons and 25,000 daltons can be seen.

RESULTS

Optimal separation of the component polypeptide chains of the Ia molecule can be achieved with a modification (8) of the buffer system first described by Laemmli (10). Figure 1 shows a comparison of the gel patterns of the Ia antigens determined by the I-A region of B10.A (*H-2^a*) when these antigens are electrophoresed in a nonstacking buffer system (11) (Fig. 1*A*) or in a stacking buffer system (7) (Fig. 1*B*). Arrows show the positions of heavy and light chain markers co-electrophoresed on the same gels. The migration of the heavy and light chain markers is clearly different in the two systems. In addition, the Ia molecules on gel A migrate as a single component of 34,000 daltons, whereas on gel B two components of 35,000 and 27,000 are separated. As shown subsequently, the stacking system allows a similar separation of guinea pig Ia polypeptide chains.

To evaluate whether different labeling methods would yield equivalent results, we analyzed biosynthetically labeled Ia antigens (^{3}H-leucine) and surface-labeled Ia antigens (^{125}I). Since the lactoperoxidase-catalyzed method results in covalent attachment of ^{125}I to the tyrosine residues on accessible surface proteins, we also examined Ia antigens that had been biosynthetically labeled with ^{3}H-tyrosine.

In the murine system, DBA/2 (*H-2^d*) spleen cells were labeled in order to study Ia molecules determined by both the I-A and I-C subregions. The results of this study are shown in Figure 2. The ^{3}H-leucine-labeled Ia molecules migrate in two discrete peaks of approximately 35,000 and 27,000 daltons. The H-2K molecules, shown for comparison, electrophorese in a single peak of slower mobility. Their apparent m.w. is 45,000. In contrast, analysis of the ^{125}I-labeled Ia molecules generated a different electrophoretic pattern. The 35,000 dalton component is poorly labeled, whereas the 27,000 dalton component is highly labeled. Since the gel pattern of the ^{3}H-tyrosine-labeled Ia molecules is similar to that of their ^{3}H-leucine-labeled counterparts, the poor labeling of the 35,000 dalton component cannot be explained by the lack of tyrosine residues.

In the guinea pig system, an additional discrepancy was noted. When ^{3}H-leucine is used as the biosynthetic label, Ia antigens can be organized into three chemical categories on the basis of molecular structure (12). Antigens of the first category (e.g. Ia.2) are composed of one 33,000 dalton chain and one 25,000 dalton chain in a noncovalent association. Thus, Ia.2 migrates in two discrete peaks under either reducing or nonreducing conditions. Antigens in the second group (e.g. Ia.4, Ia.7) are composed of a 33,000 dalton chain and a 25,000 dalton chain joined by disulfide bonds, and thus migrate under nonreducing conditions with a mobility of 58,000 daltons. Antigens in the third category (e.g. Ia.1) migrate in a single peak corresponding to a m.w. of 26,000, under reducing or nonreducing conditions.

Previous studies with ^{125}I-labeled guinea pig Ia antigens had revealed only a 25,000 dalton chain even under nonreducing conditions, and the 58,000 dalton molecule was not seen (5). In these earlier studies lentil lectin affinity chromatography had not been used to purify the antigen partially. Figure 3 shows the Ia antigens of strain 2 guinea pig lymph node cells which had been labeled with ^{3}H-leucine, ^{125}I, or ^{3}H-tyrosine, partially purified by lentil lectin affinity chromatography, isolated by immunoprecipitation, and electrophoresed under both reducing and nonreducing conditions. The antiserum used for this experiment reacts with both Ia.2 and Ia.4 antigens which are borne on separate molecules (13). With reduction (A, E, I), two peaks are seen in all instances, except that the 33,000 dalton peak is much less well labeled than the 25,000 dalton peak when ^{125}I is used. This finding confirms the results in the murine system.

When Ia antigens partially purified by lentil lectin affinity chromatography were analyzed without reduction to preserve disulfide bonds (C, G, K), the 58,000 dalton peak was readily observed with all three labeling methods. If the affinity chromatography step is not used, the 58,000 dalton peak, though

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present, is not very prominent because of the high background due to contaminants (data not shown).

In Figure 4 the Ia antigens of strain 13 guinea pigs are examined. The antiserum used precipitates a mixture of Ia.7 and Ia.1, which are borne on independent molecules (12). With reduction (panels A, E), two peaks are seen with the ^{3}H-leucine preparation, but only a single peak appears when the iodinated prepartion is examined. Here the 33,000 dalton peak from Ia.7 is apparently so poorly labeled that it is not observed. When

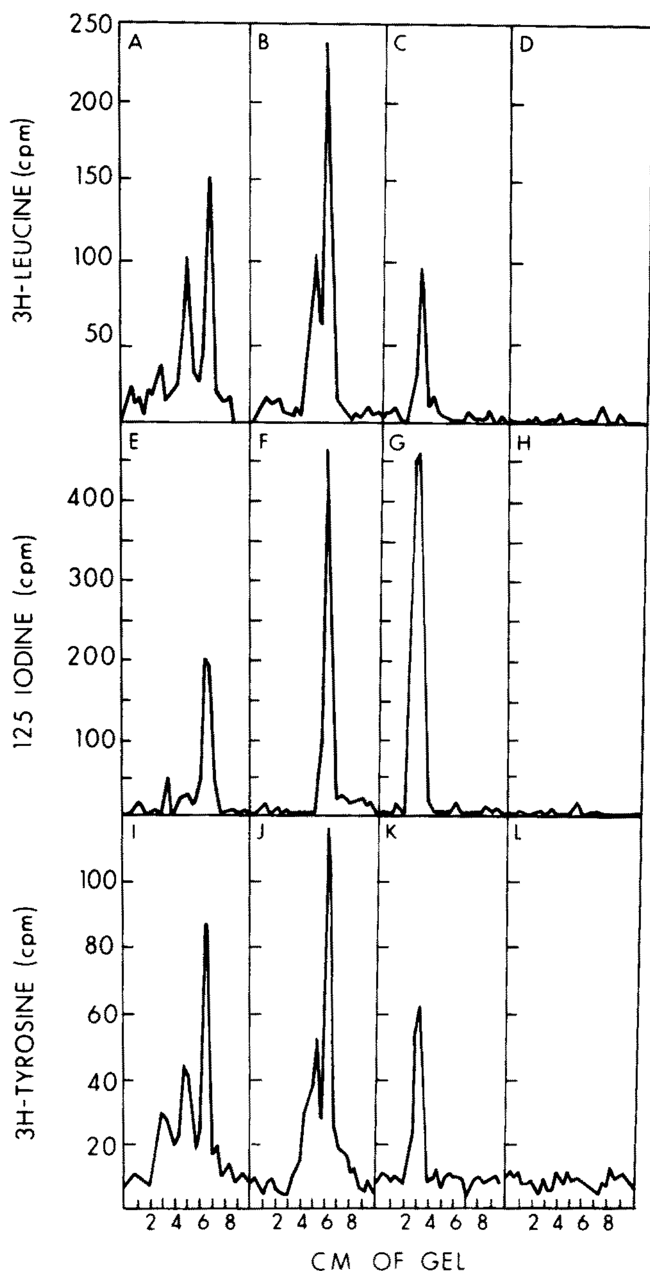

Figure 2. Electrophoretic patterns of mouse Ia and H-2 antigens labeled with ^{3}H-leucine, ^{125}iodine, or ^{3}H-tyrosine. I-A subregion-determined Ia molecules (A, E, I) precipitated by (B10 × A) F_1 anti-B10.D2 antiserum show two peaks when labeled by ^{3}H-leucine or ^{3}H-tyrosine, but the peak with slower mobility (~35,000) is not heavily labeled with ^{125}I. A small amount of H-2 antigen is detected by this serum (peaks at cm 3). I-C subregion-determined Ia molecules (B, F, J) precipitated by A.TH anti-A.TL antiserum show a similar pattern of two peaks with ^{3}H-leucine or ^{3}H-tyrosine labeling, and only a single major peak with ^{125}I labeling. The H-2 molecules (C, G, K) precipitated by B10.A anti-Meth A antiserum are shown for comparison. Controls (D, H, L) are the precipitates made with normal mouse serum.

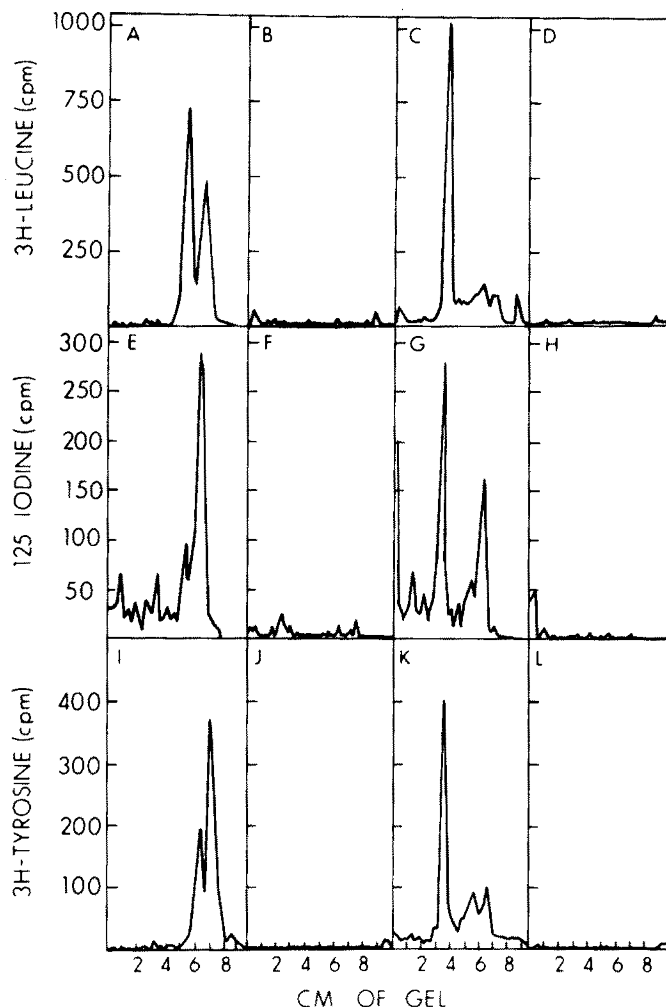

Figure 3. Electrophoretic patterns of strain 2 guinea pig Ia antigens labeled with ^{3}H-leucine, ^{125}iodine, or ^{3}H-tyrosine. A mixture of Ia.2 and Ia.4 molecules is detected by the antiserum used in these experiments. The reduced patterns (A, E, I) are shown with accompanying controls made with normal guinea pig serum (B, F, J). With ^{3}H-leucine labeling, two peaks are observed, whereas the peak with slower mobility is much less prominent with ^{125}iodine labeling. With ^{3}H-tyrosine labeling this peak is also somewhat less prominent than in the ^{3}H-leucine labeled preparation. When the precipitated material is electrophoresed without reduction (C, G, K), a larger molecular weight species is seen in all three preparations. The Ia.4 molecules are found in a 58,000 dalton peak. The ratios of counts in the 33,000 and 25,000 dalton species (Ia.2) that are also observed in these gels show that in the ^{3}H-leucine- and ^{3}H-tyrosine-labeled preparations these polypeptides are more equally labeled than in the ^{125}I-preparation, in which the 33,000 dalton species is less prominent than the 25,000 dalton. Panels D, H, L are the unreduced controls.

the unreduced gels are examined (C, G), it can be seen that the 58,000 dalton peak from Ia.7 is clearly iodinated. In addition, the Ia.1 antigen, which never displays a 33,000 dalton peak, does show labeling of the 26,000 dalton species. Thus we have shown that guinea pig Ia antigens of all three categories described can be labeled by iodination.

DISCUSSION

Identification of the 33,000 to 35,000 dalton and 25,000 to 27,000 dalton chains of which all mouse and guinea pig Ia antigens (except guinea pig Ia.1 and Ia.6) are composed has not always been achieved in the past (2-6). This paper has compared the two most widely used radiolabeling techniques

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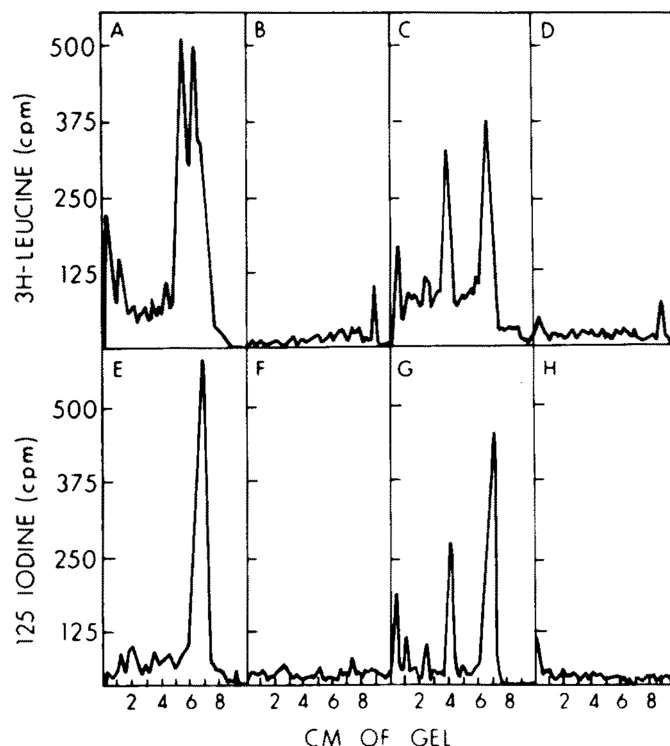

Figure 4. Electrophoretic patterns of strain 13 guinea pig Ia antigens labeled with ^{3}H-leucine or ^{125}iodine. A mixture of Ia.1 and Ia.7 molecules is precipitated by the antiserum used in these experiments. The reduced patterns (A, E) are shown with accompanying normal guinea pig serum controls (B, F). Two peaks are observed with ^{3}H-leucine, but only one with ^{125}I-labeling. Electrophoresis without reduction (C, G) shows the larger (58,000 dalton) species (Ia.7) whereas the Ia.1 molecules show only a single peak regardless of the radioactive precursor. Unreduced controls are also shown (D, H).

and presents three explanations for the discrepancies found between the two methods.

Firstly, the SDS-PAGE system used is of critical importance. A nonstacking system does not have resolving power equivalent to the stacking system, and thus may not separate two polypeptide chains that differ only by a few thousand daltons. Thus, stacking gel systems should be used to maximize separation. It should be cautioned that m.w. assigned on the basis of SDS-PAGE are only approximate, since mobility, particularly of glycoproteins, may not be linear with respect to m.w., particularly when lower concentrations of acrylamide are used (14).

The second factor accounting for the discrepancies is the choice of labeling technique. Lactoperoxidase-catalyzed radioiodination clearly labels the 25,000 to 27,000 dalton chain much better than the 33,000 to 35,000 dalton chain. One possible explanation for this finding was that the larger chain is tyrosine-poor. This explanation seems unlikely because of the observation that ^{3}H-tyrosine labels both chains almost equally well, and that with certain murine Ia specificities, the larger chain is labeled relatively well by lactoperoxidase. It is possible that the generally poor radioiodination of the 33,000 dalton chain means that the entire chain or else the majority of its tyrosine residues is sequestered (15), and not readily labeled by lactoperoxidase.

Finally, the purity of the preparation from which the Ia antigens are immunoprecipitated is critical. When ^{125}I-labeled guinea pig Ia antigens were not partially purified by affinity chromatography, the nonspecific background on the gel was sufficient to mask the presence of the 58,000 dalton molecule. The elimination of this background by passage over lentil lectin affinity columns (8) obviates this problem. On the basis of the results obtained when reducing conditions were used, it appears as if the vast majority of the ^{125}I in the 58,000 dalton species is associated with its 25,000 dalton component. Some murine Ia antigens can also be found in a 58,000 dalton form (2, 16) and these will be discussed in a subsequent paper (Cullen and Littman, in preparation).

It would appear from these experiments that use of the lactoperoxidase-catalyzed radioiodination method increases the difficulty of detecting the 33,000 chain. Since this method labels accessible surface proteins, comparison of the results from this method and from biosynthetic labeling techniques may indicate that the 33,000 dalton chain is buried more deeply in the membrane, thus providing an indication of the orientation of the molecule on the cell surface. A similar approach led to the conclusion that glycophorin was a transmembrane protein (17).

Although it is noteworthy that iodination may leave some membrane proteins unlabeled, it is clear that biosynthetic techniques also have some deficiencies. Lactoperoxidase-catalyzed radioiodination will label cell surface proteins irrespective of their turnover time, whereas biosynthetic methods will preferentially label cell proteins with rapid turnover rates. For example, radioiodination has proved superior to biosynthetic techniques in the detection of IgD (18). We would therefore conclude that no single labeling method can be definitive in every respect, and that both methods are necessary for the successful study of cell membrane molecules.

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