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Interaction of the Hinge Region of Human Immunoglobulin G with a Murine Lymphocyte Membrane Receptor

RELEVANCE TO THE PROBLEM OF ANTIGLOBULIN INDUCTION IN RHEUMATOID ARTHRITIS

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Summary. Evidence is presented which indicates the presence on murine lymphocytes of a membrane receptor for determinants on the hinge region of human IgG. These determinants are exposed following pepsin scission of the IgG molecule, i.e. on the $F(ab')_2$ fragment. The evidence for a hinge receptor derives, *in vivo*, from splenic localization of $F(ab')_2$ in germinal centres and, *in vitro*, from immunofluorescent binding studies. The sequential immunofluorescent pattern for the uptake of human $F(ab')_2$ fragments into murine spleen germinal centres was identical with that previously observed for heat-aggregated human IgG, but $F(ab')_2$ fragments appeared to be retained in the germinal centres for a shorter time than aggregated IgG. Experiments with nude mice and T cell-deprived mice showed that the localization of $F(ab')_2$ fragments does not require T cells. Competition experiments suggest that the receptor for $F(ab')_2$ may bear little relation to the receptor for aggregated IgG. The relevance of such a lymphocyte membrane receptor to the immunopathology of rheumatoid arthritis is discussed in the light of previous findings that a proportion of the serum IgG of patients with rheumatoid arthritis has a structural anomaly compared with control IgG, characterized by exposure of new determinants at the hinge region.

INTRODUCTION

The existence of Fc receptor on B lymphocyte membranes specific for heat-aggregated IgG and immune complexes has been demonstrated (Dickler and Kunkel, 1972; Basten, Miller, Sprent and Pye, 1972). Both aggregated IgG and immune complexes are also antigens for rheumatoid factor antibody (Henney and Stanworth, 1965).

We have recently demonstrated the presence of 'conformationally-altered' IgG in monomeric, native serum IgG of patients with rheumatoid arthritis (Johnson, Watkins, Scopes and Tracey, 1974; Watkins and Swannell, 1972) and our evidence suggests that the alteration in the IgG molecule is located at, or near, the hinge region of the molecule. The determinants exposed on the 'altered' monomeric IgG molecule have been demon-

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strated by whole body elimination studies to resemble some of the determinants exposed following pepsin scission of the IgG molecule, i.e. on the $F(ab')_2$ fragment (Watkins, Roberts and Johnson, 1975). If this conformationally altered material is an immunogen for antiglobulin production we postulated that a B-lymphocyte membrane receptor may exist that is specific for 'hinge' determinants of human IgG. We present evidence indicative of such a receptor on normal murine spleen cells which has been obtained by two techniques: *in vitro*, indirect immunofluorescence on isolated living cells; *in vivo*, splenic localization of human $F(ab')_2$ in germinal centres. The localization of heat-aggregated IgG and immune complexes in germinal centres of normal mouse spleens has been described in detail (Brown, Harris, Papamichail, Sljivic and Holborow, 1973; Sordat, Sordat, Hess, Stoner and Cottier, 1970) and Herd and Ada (1969) have demonstrated localization in rat lymph node of Fc fragments but not Fab fragments of rabbit IgG. The uptake of human $F(ab')_2$ by lymphocytes and its localization in germinal centres has not been previously reported.

MATERIALS AND METHODS

Mice

C57Bl, BALB/c and CBA mice of both sexes, between 2 and 6 months old, from inbred colonies at Taplow were used in these studies. Congenitally athymic mice, homozygous for the mutation 'nude' (*nu/nu*), were obtained from the Laboratory Animal Centre, Carshalton, Surrey. CBA mice that had been thymectomized, lethally X-irradiated and bone marrow-reconstituted were kindly provided by Dr E. Leuchars of the Chester Beatty Institute, London.

Immunoglobulins and fragments

Fragments of Kabi Cohn fraction II IgG, Fab and $F(ab')_2$, were generously donated by Dr M. W. Turner of the Institute of Child Health, London (Okafor, Turner and Hay, 1974; Turner, Mårtensson, Natvig and Bennich, 1969). Human Fc fragment from an IgG1 myeloma preparation was kindly supplied by Dr D. R. Stanworth of Birmingham University (Stewart, Hunneyball and Stanworth, 1975).

Serum IgG was isolated from three healthy individuals and from three rheumatoid patients as described previously (Johnson *et al.*, 1974). Individuals were chosen as clearly possessing either 'rheumatoid' or 'normal' IgG by a previous conformational analysis of their IgG using circular dichroic spectra (Johnson *et al.*, 1974).

Heat-aggregated IgG was prepared as described previously (Greenwood, Brown, de Jesus and Holborow, 1971). Heat-aggregated Fab fragments were prepared similarly.

Radioisotope procedures

Immunoglobulin materials were labelled with ^{125}I(Radiochemical Centre, Amersham) by the iodine monochloride technique of McFarlane (1958). All mice receiving radio-iodinated material were given iodide water to block thyroid uptake of radio-iodine before and during the experiment. Mice were killed at various times after administration of radiolabelled material and their spleens removed and weighed. The radioactivity in the spleen was counted in a Tracerlab Gammaguard 150 spectrometer.

Administration of immunoglobulin preparations

The immunoglobulin preparations were centrifuged at 3000 rev/min and the solution

was passed through a sterile Millipore filter (0·45 μm) immediately prior to intravenous or intraperitoneal injection into a tail vein. All materials were injected in a volume of 0·2 ml sterile saline.

Cell suspension medium

Medium 199 (BDH, Poole) supplemented with 10 per cent foetal calf serum (Bio-cult, Glasgow) was used throughout.

Preparation of mouse spleen cell suspension

C57Bl mice were killed by cervical dislocation and the spleens removed and teased through a fine platinum mesh into medium. Complete teasing of the cells was achieved by passage through a lint gauze and repeated aspiration. The cells were then layered onto a mixture prepared by adding 9·6 ml of 9 per cent Ficoll (Pharmacia, Sweden) to 4 ml of 34 per cent Hypaque (Winthrop, Surbiton, Surrey) and centrifuged at 1500 ***g*** for 25 minutes at laboratory temperature. The resultant erythrocyte-free layer of cells at the interface was aspirated and washed three times in medium.

Immunofluorescent staining

Spleen sections. Mouse spleens, following removal, were immediately snap-frozen in isopentane cooled in liquid nitrogen using 'Tissue-Tek' O.C.T. compound (Ames Company, Division Miles Laboratories, Indiana) as embedding medium. Sections were cut at 6 μm, dried under a fan and stained either directly with a specific fluorescein-conjugated sheep antiserum to human IgG or indirectly with sheep antiserum to human IgG followed by fluorescein-conjugated anti-sheep immunoglobulin antiserum. All antisera were obtained from Wellcome Reagents Limited and conjugates were used at dilutions which did not stain spleen sections from uninjected mice.

Cell suspensions. Immunofluorescent staining of the membranes of mouse spleen cells to detect binding were carried out in duplicate using 3×10^6 cells per tube. After centrifugation, the cells were resuspended in 0·2 ml of phosphate-buffered saline (PBS), pH 7·2, containing 100 μg of test material and incubated for 30 minutes at 4°. Cells were then washed in medium and resuspended in the appropriate fluorescein conjugate which had been absorbed (1:2) with normal C57Bl mouse serum. After incubation for 30 minutes at 4° the cells were washed three times in medium, resuspended in 50 per cent glycerol and mounted on slides. A minimum of 200 cells per preparation were counted.

RESULTS

C57Bl or BALB/c mice were injected with 200 μg of each immunoglobulin preparation and the results of immunofluorescence studies for germinal centre localization on sections taken from spleens that had been removed at various times after injection are summarized in Table 1. No significant differences were observed between sexes, modes of injection (i.p. or i.v.) and direct or indirect immunofluorescence. Both strains of mouse were used for these groups except for those receiving 'normal' or 'rheumatoid' IgG when female C57Bl mice were used throughout because of their discriminant immune responses to these two forms (Watkins, Turner and Roberts, 1972).

Human Fab fragments (native or heat aggregated) showed no localization in germinal centres, but in contrast both human $F(ab')_2$ and Fc localized. The intensity of immuno-

TABLE 1

MICE DEMONSTRATING SPECIFIC SPLENIC LOCALIZATION OF IMMUNO-GLOBULINS OR FRAGMENTS (POSITIVE RESULTS ARE EXPRESSED AS FRACTIONS OF TOTAL NUMBER TESTED)

Material injected	Time after injection							
	15 minutes	2 hours	4 hours	6 hours	24 hours	7 days	14 days	21 days
Human Fab	—	0/3	—	—	0/3	—	0/3	—
Human $F(ab')_2$	4/4	10/10	3/3	7/7	9/10	2/3	4/8	1/4
Human Fc	—	—	3/3	—	3/3	—	3/3	—
'Normal' human IgG	—	0/3	—	—	0/12	0/3	0/3	0/3
'Rheumatoid' human IgG	—	0/3	—	—	6/12	1/1	2/3	1/3
Aggregated human IgG	4/4	10/10	3/3	7/7	10/10	3/3	8/8	4/4
Aggregated human Fab	—	0/3	—	—	0/3	—	—	—

fluorescence due to Fc fragments localized in germinal centres was very weak, in contrast to the much stronger staining of localized human $F(ab')_2$ fragments. The sequential pattern for the uptake of $F(ab')_2$ fragments was identical with that for heat-aggregated IgG. For example, after 15 minutes some fluorescent material could been seen in the red pulp and marginal zones, after 2 hours the fluorescence was distributed between the marginal zone and mantle layer of the white pulp and by 6 hours most of the material had localized in the germinal centres, giving the characteristic dendritic pattern of staining as shown in Fig. 1a. At times longer than 24 hours, however, the retention of $F(ab')_2$ fragments in germinal centres was less than that of aggregated IgG.

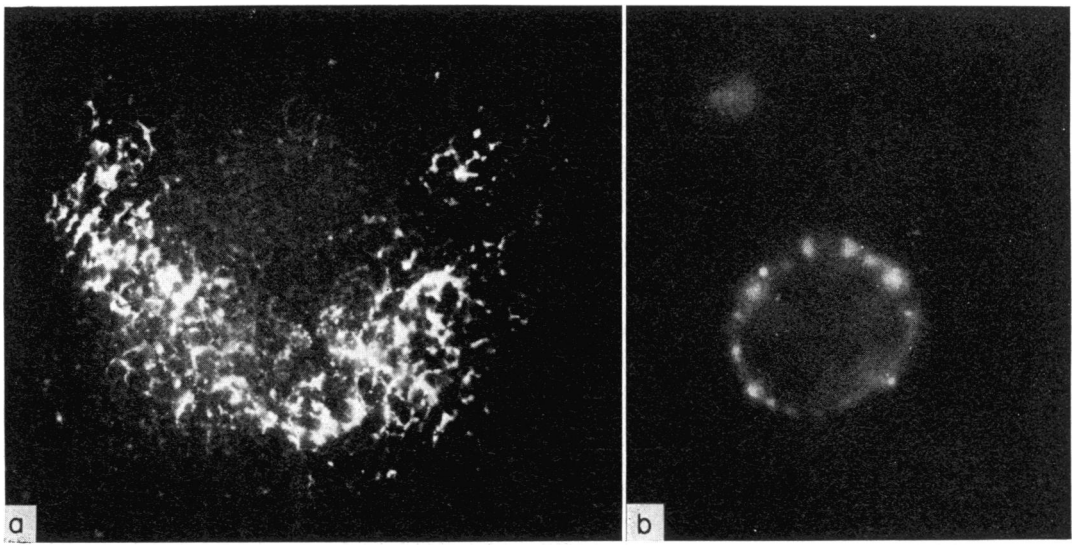

FIG. 1. (a) Cryostat section of mouse spleen taken 24 hours after i.p. injection of 200 μg human $F(ab')_2$ and stained with fluorescein-conjugated anti-human IgG. (Magnification × 400.) (b) Mouse spleen cell after incubation with aggregated human IgG and stained with fluorescein-conjugated anti-human IgG. (Magnification × 1200.)

Experiments were also performed with rabbit $F(ab')_2$ kindly provided by Dr D. R. Stanworth, but the results with this material (not shown in Table 1) were inconclusive. Spleens of mice taken 4 or 24 hours after injection of rabbit $F(ab')_2$ and stained with anti-rabbit immunoglobulin–FITC, showed an increased intensity of fluorescence in the white pulp, notably in the marginal zone, as compared with uninjected mice but there was no definite germinal centre staining.

Freshly isolated normal human serum IgG (200 μg) showed no localization in the germinal centres. Similarly Kabi Cohn fraction II IgG did not localize, despite the high proportion (approximately 10 per cent) of dimer IgG in such a commercial preparation. However, the same dose of a freshly isolated rheumatoid serum IgG showed definite, though weak, germinal centre localization in some mice. We have previously demonstrated that approximately 50 per cent of female C57Bl mice exhibit an immune response against 'rheumatoid' IgG after 7 days, whereas they all appear tolerant to 'normal' IgG (Watkins *et al.*, 1972). Hence those mice showing localization of rheumatoid IgG at or after day 7 (Table 1) could be exhibiting specific antibody-dependent germinal centre localization. Nevertheless, it is interesting to note that some mice localized the rheumatoid material in splenic germinal centres as early as 24 hours indicating an antibody-independent mechanism for the germinal centre localization of this material.

Congenitally athymic, nude, mice were able to localize $F(ab')_2$ within 24 hours in the characteristic dendritic pattern in the splenic white pulp. The existence of morphological germinal centres in spleen sections from these mice was shown histologically by staining with haematoxylin and eosin, and also by silver impregnation of the reticulin framework. Similarly T cell-deprived CBA mice were found to localize $F(ab')_2$ within 24 hours in splenic germinal centres, as did control normal CBA mice.

The results of *in vivo* competition for localization in the germinal centres are summarized in Table 2. C57Bl mice were injected with 400 μg of the unlabelled material and, after an appropriate time, injected with 50 μg of the radioiodinated material. Mice were killed

TABLE 2

In vivo COMPETITION EXPERIMENT

Unlabelled material (400 μg)	Radioiodinated material (50 μg)	Time between injections	Percentage uptake after 24 hours of radioiodinated material per milligram of spleen weight compared to control mice receiving only the radioiodinated material*
Aggregated IgG	$F(ab')_2$	10 minutes	84
Aggregated IgG	$F(ab')_2$	1 hour	61
Aggregated IgG	$F(ab')_2$	2 hours	68
Aggregated IgG	$F(ab')_2$	4 hours	61
Aggregated IgG	$F(ab')_2$	24 hours	58
$F(ab')_2$	Aggregated IgG	10 minutes	87
$F(ab')_2$	Aggregated IgG	1 hour	79
$F(ab')_2$	Aggregated IgG	2 hours	92
$F(ab')_2$	Aggregated IgG	4 hours	81
$F(ab')_2$	Aggregated IgG	24 hours	73
Fab	Aggregated IgG	1 hour	93
Fab	Aggregated IgG	1 hour	94
Monomeric IgG	Aggregated IgG	1 hour	99
Monomeric IgG	Aggregated IgG	2 hours	97

* Figures quoted are the average of two or three mice tested at each interval. The variation in counts between individual mice in any group did not exceed 10 per cent.

24 hours after the second injection and the percentage uptake of radioiodinated material compared with that in control mice receiving only the radioiodinated material is shown in Table 2. A 30–40 per cent reduction in splenic uptake of radioiodinated $F(ab')_2$ was observed in mice previously receiving unlabelled aggregated IgG. In the converse situation where mice had first received $F(ab')_2$ the uptake of radioiodinated IgG was reduced only by about 15 per cent. These results indicate some competition between the two preparations, but point to the likelihood that the aggregated IgG has greater avidity for the receptor involved than $F(ab')_2$.

In vitro immunofluorescent studies to demonstrate specific $F(ab')_2$ binding were carried out on erythrocyte-free living mouse spleen cell suspensions. The results, both for test and control cells, are summarized in Table 3. The proportion of cells in the suspension showing surface staining with anti-mouse immunoglobulin–FITC was 68 per cent: this figure is higher than might be expected, perhaps because of a selective enrichment of the B-cell population by the Ficoll–Hypaque separation technique. Virtually no binding of Fab fragments was seen, whereas 33 per cent of the cells bound 'normal' IgG and 55 per cent bound 'rheumatoid' IgG. However, the intensity of fluorescence for these test materials was weak. $F(ab')_2$ fragments gave a greater intensity of fluorescence and 63 per cent of the

TABLE 3

IMMUNOFLUORESCENCE ON C57Bl MICE SPLEEN CELLS

First layer	Second layer	Intensity of fluorescence	Percentage of positively staining cells
Fab	Anti-human Ig–FITC	+	1·9
$F(ab')_2$	Anti-human Ig–FITC	++	63
'Normal' IgG	Anti-human IgG–FITC	+	33
'Rheumatoid' IgG	Anti-human IgG–FITC	+	55
Aggregated IgG	Anti-human IgG–FITC	+++	73
IgA	Anti-human IgA–FITC	++	4·1
PBS	Anti-human Ig–FITC	+	1·7
PBS	Anti-human IgG–FITC	–	0
PBS	Anti-human IgA–FITC	–	0
PBS	Anti-mouse Ig–FITC	+++	68

cells showed positive staining. Although the staining for bound $F(ab')_2$ was less intense than for aggregated IgG, it was clearly brighter than that seen with monomeric IgG. All positive staining of spleen cells after treatment with human IgG (native or aggregated) or its fragments was irregular and speckled in distribution on the cell membrane with no evidence of cap formation (Fig. 1b). This was quite distinct from the ring pattern seen with anti-mouse immunoglobulin–FITC.

In vitro competition experiments between $F(ab')_2$ and heat-aggregated IgG for murine spleen cell receptors were performed using radioiodinated material. It was found that the specific binding of radiolabelled aggregated IgG was not significantly reduced if the isolated spleen cells (3×10^6 cells per tube) had been previously incubated for 30 minutes at 4° with 500 μg of unlabelled $F(ab')_2$ or monomeric IgG in 0·2 ml of PBS. Similarly, there was no significant reduction of binding of radiolabelled $F(ab')_2$ if the cells had been previously incubated with 500 μg of unlabelled aggregated IgG or monomeric IgG. These experiments were repeated twice, each time in duplicate, with washing of the cells after each incubation. There was never less than 80 per cent uptake of radioiodinated material after the cells had been previously incubated with unlabelled material.

DISCUSSION

These results may be discussed: (a) in relation to the mechanism of localization of $F(ab')_2$ in germinal centres; (b) in relation to the murine lymphoid cell receptors involved; (c) as to their relevance to the induction of antiglobulin immune responses in human rheumatoid arthritis.

(a) We have demonstrated that the Fab fragment of human IgG does not localize in the germinal centres of mouse spleen, whereas $F(ab')_2$ fragments localize unequivocally. Since heat-aggregated Fab fragments also did not localize, we conclude that polymerization of the Fab fragments is not a requirement for germinal centre trapping and that the localization of human $F(ab')_2$ is mediated through determinants exposed at the hinge following enzymic scission. The failure of rabbit $F(ab')_2$ to localize could be due to steric hindrance of determinants at the hinge region by carbohydrate groupings known to be bound to this part of rabbit IgG (Smyth and Utsumi, 1967). Similarly the comparatively weak localization of human Fc fragments may represent the exposure of some 'hinge' determinants in a proportion of these fragments.

The sequential pattern of immunofluorescence staining for the uptake of human $F(ab')_2$ fragments in murine spleen germinal centres was identical with that for heat-aggregated IgG reported previously by Brown *et al.* (1973). This suggests a similar mechanism of transport to germinal centres, dependent on uptake on the surface membrane of lymphoid cells in the mantle layer of the Malpighian bodies. The experiments with congenitally athymic, nude, mice showed definite splenic localization of $F(ab')_2$ in the characteristic dendritic pattern; similar results were obtained with T cell-deprived mice. Thus the mechanism of localization of $F(ab')_2$ appears to be T cell-independent, as was reported for the localization of heat-aggregated IgG (de Jesus, Holborow and Brown, 1972). However, an immunofluorescence study of BALB/c and C57Bl mouse spleens removed at intervals longer than 24 hours after injection of $F(ab')_2$ showed that $F(ab')_2$ is retained in the germinal centres for a shorter time than aggregated IgG (Table 1).

The germinal centre localization of heat-aggregated IgG has been shown to be C3-dependent (Papamichail, Gutierrez, Embling, Johnson, Holborow and Pepys, in preparation). Preliminary experiments with mice decomplemented with purified cobra venom factor, kindly supplied by Dr M. B. Pepys of the Royal Postgraduate Medical School, London, have shown that C3 also appears necessary for uptake of $F(ab')_2$ in germinal centres. It might be suggested that $F(ab')_2$ is localized in germinal centres by a C3-dependent process involving immune complex formation with preformed mouse antibody. We could not support this hypothesis, since our data shows binding of $F(ab')_2$ to murine spleen cells and no circulating antibody to human $F(ab')_2$ could be detected in normal C57Bl mouse serum by haemagglutination of $F(ab')_2$-coated sheep red cells or by double radial immunodiffusion in agar gels. Also, circulating immune complexes could not be detected in the sera of mice 2 or 4 hours after injection of human $F(ab')_2$, using the technique of Onyewotu, Holborow and Johnson (1974).

(b) The immunofluorescence studies reported here on isolated living murine spleen cells demonstrated definite increased cell membrane binding of $F(ab')_2$, as compared with monomeric IgG or Fab fragments. This, together with the observation that $F(ab')_2$ localizes in germinal centres, points to the existence of a murine B-lymphocyte membrane receptor for determinants on the hinge region of human IgG. It is known that there is a receptor on mouse B lymphocytes specific for the Fc region of IgG bound in an immune complex

(Paraskevas, Lee, Orr and Israels, 1972), but no definitive evidence has been forthcoming that the membrane receptor binds the same region of the molecule when binding heat-aggregated IgG. Indeed, different reports have conflicted in suggesting whether the binding of aggregated IgG by human B lymphocytes is dependent or independent of Fc receptors (Dickler, 1974; Froland, Natvig and Michaelsen, 1974). Heat aggregation of IgG might be expected to expose new determinants in the hinge region of the IgG molecule and hence some binding could be mediated through a hinge-specific membrane receptor. Nevertheless our competition experiments, both *in vivo* and *in vitro*, tend to suggest that the receptors for aggregated IgG and for $F(ab')_2$ may differ.

Thus it would appear from our experiments and from other published results that IgG binding to B lymphocytes may be heterogeneous, involving more than one site of interaction on the IgG molecule and that these may have different binding affinities.

(c) We have previously demonstrated the presence of 'conformationally altered' IgG in the serum of patients with rheumatoid arthritis (Johnson *et al.*, 1974; Watkins and Swannell, 1972). These studies suggested that new determinants are exposed at or near the hinge region of the rheumatoid IgG molecule and that these resemble those exposed on the $F(ab')_2$ fragment following pepsin scission of the intact molecule (Watkins *et al.*, 1975). It is thus interesting to observe that rheumatoid IgG exhibits a tendency to behave like $F(ab')_2$ fragments in both the *in vivo* and *in vitro* test systems described in this paper. It must be emphasized that for the present work individuals were chosen who clearly possessed either 'rheumatoid' or 'normal' IgG as determined by conformational analysis of their serum IgG using circular dichroism (Johnson *et al.*, 1974). The reason for production of conformationally altered IgG in the rheumatoid patient is unknown. However, our data indicate that it expresses an increased binding to murine B cells, as compared with normal IgG, and this increased binding could favour the stimulation of those cells which carry a specific receptor for an antigenic part of the molecule in the Fc region. In this way an accumulation of 'rheumatoid' IgG in the body might break tolerance and result in antiglobulin production and immune complex formation, both of which are associated with the pathogenesis of rheumatoid disease. The breaking of specific tolerance would reflect the extent of conformational alteration in the rheumatoid serum IgG population. However, mechanisms such as proteolytic degradation of IgG by cathepsins in normal catabolism can produce fragments closely resembling $F(ab')_2$ (Lo Spalluto, Fehr and Ziff, 1969) and it may be misleading to infer in the rheumatoid patient the possibility of a lymphocyte response exclusively to autologous, monomeric IgG. Nevertheless, it is interesting to note that follicular hyperplasia is often a feature of rheumatoid lymphoid tissues (Cruickshank, 1958). A detailed analysis of the interaction between lymphoid cells and 'altered' IgG or IgG fragments with regard to immunogenicity and antiglobulin specificity is clearly needed.

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