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Heat inactivation of the neuraminidase and haemagglutinin estimated in the agglutination–separation reactions using red blood cells sensitized with Newcastle disease virus

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Abstract – The agglutination–separation (AS) reactions estimate the effects of heat on the release of altered Newcastle disease virus (NDV) and HN glycoprotein spikes from red blood cells (RBC) sensitized with NDV (SRBC), the inactivations of the neuraminidase (NA), then the haemagglutinin (HA) in a direct assay. Heating SRBC for 1.5 min at 56 °C inactivated the NA by 50 %; after 4.5 min no separation occurred indicating 100 % inactivation of the NA. Heating a suspension of NDV for 78 min inactivated the NA 50 % as assayed by cleavage of fetuin. Comparatively, the AS test was up to 52-fold (78 min/1.5 min = 52) more efficient in detecting NA inactivation than was the basic reference test where cleavage of fetuin was assayed. The HA was 50 % inactivated after 18 min of heating and 100 % inactivated after 36 min as no agglutination was seen. Free HA on SRBC was agglutinated by and thus was titrated with the sialic acid on NRBC. The large area of RBC increased the efficiency of the AS test when compared with tests using suspensions of NDV. At 51–60 °C all NA and HA inactivations were sequential, and invariably the NA was more heat labile than the HA. The release of altered NDV and HN spikes was inhibited with mild heat although the separation of SRBC and NRBC continued. Biological purifications showed that the heat stability of the HA and the lability of the NA were genetically stable. © Inra/Elsevier, Paris.

Newcastle disease virus / neuraminidase / haemagglutinin / agglutination–separation reaction / sequential heat inactivation

Résumé – Inactivation par la chaleur de la neuraminidase et de l’hémagglutinine estimée dans les réactions d’agglutination-séparation en utilisant des héméries sensibilisées avec le virus de la maladie de Newcastle. Les réactions d’agglutination-séparation (AS) permettent d’estimer l’effet de la chaleur sur la libération de virus de la maladie de Newcastle (NDV)

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modifié et des spicules de glycoprotéines HN à partir d’hématies sensibilisées avec le virus, l’inactivation de la neuraminidase (NA), puis de l’hémagglutinine (HA) dans un test direct. Le chauffage des hématies sensibilisées pendant 1,5 min à 56 °C a inactivé la NA de 50 % ; après 4,5 min, aucune séparation ne s’est produite, indiquant une inactivation de 100 % de la NA. Le chauffage d’une suspension de NDV pendant 78 min a inactivé la NA de 50 %, ce qui a été vérifié par le test de clivage de la fétuine. Le test d’AS était donc jusqu’à 52 fois plus efficace (78 min / 1,5 min = 52) pour la détection de l’inactivation de NA que le test de référence basé sur le clivage de la fétuine. L’hémagglutinine était inactivée de 50 % après 18 min de chauffage et était inactivée de 100 % après 36 min, sans agglutination observée. La HA libre sur les hématies sensibilisées était agglutinée et a été titrée par de l’acide sialique sur des hématies normales. La grande surface des hématies a augmenté l’efficacité des tests AS comparativement aux tests utilisant des suspensions de NDV. À 51–60 °C, toutes les inactivations NA et HA étaient séquentielles, et immanquablement la NA était plus sensible à la chaleur que l’HA. La libération de NDV modifié et de spicules de HN était inhibée par un chauffage doux, bien que la séparation des hématies sensibilisées et normales persistait. Les purifications biologiques ont montré que la stabilité à la chaleur de l’HA et la labilité de NA étaient génétiquement stables. © Inra/Elsevier, Paris.

virus de la maladie de Newcastle / neuraminidase / hémagglutinine / réaction d’agglutination-séparation / inactivation par chauffage séquentiel

1. INTRODUCTION

Mild heat applied to suspensions of Newcastle disease virus (NDV) inactivated the elution-inhibition (EI) antibody determinant. In the presence of EI antibody the neuraminidase (NA) was then functional and NDV eluted from red blood cells (RBC). Additional heat partially inactivated the NA as shown by a reduced rate of elution. Partial inactivation of the haemagglutinin (HA) required more heat as assayed in HA inhibition (HI) and HA titrations. Thus, three assay systems were required to rank the heat labilities in the following order: EI determinant > NA > HA [14].

Responding to EI as well as HI antibody in polyclonal avian antisera was the initial indication that the HA and NA both exist [12, 29], now an established concept for NDV [22]. Populations have been tested for EI antibody with acceptable accuracy [15]. Moreover, the heat lability of the NA suggested that the HA and NA domains could be individually examined if an appropriate assay was available. Ultimately the strain differences referable to the HA or NA may be quantified and correlated with known sequences or, eventually, the atomic structures [8, 21, 30].

The present objective is to describe an efficient assay for estimating the heat labilities of the NA and HA within a single system. To verify the results derived from the three assays and to further quantify the NA and HA inactivations, the effects of heat were estimated in the agglutination–separation (AS) reactions.

The AS reactions use normal RBC to agglutinate RBC sensitized with NDV. Incubation resulted in the separation of the agglomerate of sensitized and normal RBC. Thus, the respective HA and NA activities are estimated by the agglutination followed by the separation of the RBC. The AS test allows visualization of each inactivation in a direct, single system, thus providing a method to separate and sequentially inactivate both activities associated with the HN glycoprotein spike (HN spike) [18]. The comparison of the heat labilities of the NA and HA estimated in one assay system would be internally consistent and hence more accurate.

The inactivations of the NA then the HA at selected times and temperatures are herein reported. The results of the AS tests are compared with those from tests which use suspensions of NDV. An efficient test for the
NA has been developed. Structure–function relationships are considered based on other studies and the heat labilities estimated with the AS reactions.

2. MATERIALS AND METHODS

2.1. Strain 575 NDV and RBC

Strain 575 (PMV-I/Mute swan/Maryland/575/1977) has been described \[14, 15\]. Selected leghorn chicken RBC were washed and suspended at 0.3 % concentration in phosphate (0.05 M) buffered (pH 7.4) NaCl (0.14 M) (PBS) containing 0.1 % NaN\(_3\). Screening for appropriate RBC from donor chickens is necessary since, for unknown reasons, certain RBC do not sensitize properly \[14\].

2.2. Biological purification of strain 575 by terminal end point dilution and plaque isolations

The initial 575 isolate showed the EI reaction with polyclonal antisera if sufficient HA units were available in the test \[12, 14\]. The second egg passage was diluted until reaching the terminal dilution of 10\(^9\). Allantoic cavities of 11-day-old embryonated eggs were inoculated with diluted NDV and harvested after 72 h of 37 °C incubation. No embryo deaths were noted. NDV was biologically cloned by plaque purification in chicken embryo cell monolayers \[11\]. Following growth in eggs the plaque purification was repeated. Purified NDV from egg passages at levels three to five were used for the heat inactivations, the AS reactions and other tests using suspensions of NDV \[14\].

2.3. Heating sensitized RBC

Sensitized RBC \[5\] in PBS haemolysed and formed ghosts when heated for 1 h at 51 °C, 5 min at 53 °C or immediately at 56 °C. The ghosts retained the HA and NA activities indicating that of the three known sialic acid–HA linkages \[9, 25, 26, 28\] those present were not cleaved by heating the sensitized RBC. Thus, with mild heat the cleavage of the HA–sialic acid bond is not involved in the sequential inactivations. The free HA and NA of the NDV and perhaps the HN spikes are located on the sensitized RBC.

Many are exposed and available for heat inactivation \[14\].

Sensitized RBC at 0.3 % concentration were brought from 25 °C to the desired temperature in a water bath with a thermometer placed directly in the vessel. After 4 min in the water bath a temperature of 56 °C was attained. For optimal accuracy in comparing the NA and HA inactivations the rise in temperature should be rapid while heating sensitized RBC to 56 °C. Aliquots were removed and were used in the AS tests. The tests were conducted with 0.025 mL volumes in microtitre plates.

2.4. Agglutination–separation reactions and test

The free HA on sensitized RBC can be titrated with normal RBC. In the AS test one volume of sensitized RBC agglutinates up to seven volumes of normal RBC. The HA bonds with sialic acid on normal RBC. With incubation the NA is reactivated, the agglomerate of normal and sensitized RBC separate, and at some period the altered NDV and HN spikes are released. The proportion of altered NDV/HN spikes is governed by the amounts of sialic acid on the normal RBC in the AS reaction. Thus, the HA substrate is the HA–sialic acid bond rather than added artificial compounds such as fetuin. The AS reactions are irreversible since altered NDV and HN spikes can be separated on sephadex G-200 \[14\] under non-reducing conditions (unpublished results).

If, after heating the sensitized RBC and mixing with normal RBC, the agglomerates in wells 1–3 or 4 remained agglutinated, the HA was considered to be 50 % inactivated. No agglutination indicated 100 % inactivation. After the agglutination end points were determined, the tests were incubated at 37 °C in a humidified atmosphere to assess the separation of the agglomerates.

The time of incubation was not set since heated sensitized RBC, like the elution from RBC using suspensions of heated NDV \[14\], show a reduced rate of separation of the agglomerate. The inactivation of the HA was assessed when no further separation of the agglomerate was noted. End points were usually reached after 16 to 48 h of incubation. If separation occurred in all seven wells the end point was scored < 1/1. If the agglomerates in wells 3 or 4–7 separated and formed pellets of RBC, the NA was considered to be 50 % inactivated. No separation of the agglutinated RBC in wells 1–7 indicated 100 % inac-
tivation of the NA. Evaluation of partial, although permanent, separation or agglutination in wells 3 and 4 was often possible, thus allowing greater precision in the estimation of end points. Such end points were scored +/-.

2.5. Heat inactivation of the NA and HA activities in suspensions of NDV

A suspension of NDV containing 16 HA units per 0.1 mL of PBS was heated to 56 °C. Duplicate 0.1-mL aliquots were removed and incubated at 37 °C for 70 min or 17 h with 1.25 mg fetuin (Sigma Chemical Corporation, Saint Louis, MO) in 0.1 mL PBS. The cleavage of sialic acid from fetuin was estimated in the standard assay [2]. The optical density (OD) of the reaction product was measured at 549 nm.

Diluted suspensions of strain 575 were heated and tested for the decline in HA content by reacting with RBC in the HA assay. The increase in the HI antibody titre was also used to assess when the HA was inactivated. The NA activity was assessed by the rate of elution [14]. The time required for 50 % inactivation of the HA and NA was used for comparison with both the AS and cleavage of fetuin tests.

If 100 % inactivation was not directly measured, rather than using extrapolated values, the time for partial inactivation was used in comparisons with other tests. These comparisons are expressed as greater than (>) or less than (<). If both values were > or < the comparison is expressed as (+/-). If sufficient observations are made after heating, an AS test will exhibit a 50 or 100 % inactivation end point. Still, it is not known when that precise end point occurred during heating of the sensitized RBC. The observation point prior to the end point in question was used to calculate the mean of the heating times before and at 50 or 100 % inactivation. This mean time was a working estimate of the time required to inactivate the NA or HA. The times were used to compare the HA and NA at 50 or 100 % inactivation with the results from other tests described below.

2.6. Release of altered NDV and HN spikes from sensitized RBC

To detect the effects of heat on the release of altered NDV and HN spikes from agglomerates which had separated, the fluids from the AS tests were removed and dispensed in a microtitre plate. From a 0.15 % suspension of RBC, 0.025 mL was added to the microtitre plates. Since the NA was more heat labile than the HA, the agglutination assay for heated HA would have functioned if released altered NDV and HN spikes were present even if the NA was inactivated. This consideration is only for purposes of clarification. As shown below, to release altered NDV and HN spikes in the AS reactions a functional NA is required.

3. RESULTS

3.1. The 50 and 100 % inactivation of the HA and NA activities estimated in the AS test

The time and temperature of inactivation of the NA and HA on sensitized RBC at 51–60 °C showed that the NA activity was always more labile than the HA activity; the time required for inactivation decreased as the temperature increased (table I). The HA was not influenced by terminal dilution or plaque purifications. Purified NDV from passage levels 3 to 5 gave similar heat-inactivation results.

At 53 °C the agglomerates separated and altered NDV and HN spikes were released.
At this time separation was noted in wells 4–7 and HA were released in wells 5–7; after 3 min of heating at 56 °C, separation was noted in well +/–5 to well 7. Thus, a mean of 1.5 min (0–3 min) was required for 50 °C inhibition of the NA; no separation was apparent after a mean of 4.5 min (3–6 min) of heating, indicating 100 °C inactivation of the NA. No altered NDV and HN spikes were released between 0 and 3 min, although separation continued at 3 min in wells ± 5–7 (table I).

In another experiment at 53 °C complete separation of agglomerates and release of altered NDV and HN spikes continued up to 50 min; at 60 min separation in wells ± 4–7 was seen indicating a mean of 55 min for 50 °C inhibition of the NA (table I). Release ceased at 50 min. Thus, at 53 °C as at 56 °C release of altered NDV and HN spikes is more heat labile than separation of the agglomerates (table II).

At 56 °C the release was apparent in tests at 7/1 to 5/1 but not at the lower ratios of normal to sensitized RBC. The release in the AS reactions is enhanced with increased concentrations of sialic acid present on the normal RBC (table II); thus, titrations for the released altered NDV and HN spikes are more accurate if fluid from the 7/1 test is used.

To summarize, the NA can be assessed at 51, 53 and 56 °C, and the HA at 56, 58 and 60 °C (table I). Both the NA and HA activities can be estimated at 56 °C; at 50 and 100 °C inactivation the NA was 12-fold (18 min/1.5 min = 12) and 8-fold (36 min/4.5 min = 8) more labile, respectively, than the HA. Comparisons with other tests appear in tables I and III.

### Table I. The temperatures and time (min) required for 50 or 100 % inactivation of the neuraminidase (NA) and haemagglutinin (HA) activities on red blood cells (RBC) sensitized with Newcastle disease virus in agglutination-separation tests.

<table>
<thead>
<tr>
<th>Temperature (°C) of heating sensitized RBC</th>
<th>Separation (NA)</th>
<th>Agglutination (HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>50 %b</td>
<td>100 %</td>
</tr>
<tr>
<td>53</td>
<td>50–60 (55)</td>
<td>&gt;60</td>
</tr>
<tr>
<td>56</td>
<td>0–3c (1.5)</td>
<td>3–6 (4.5)</td>
</tr>
<tr>
<td>58</td>
<td>0–10 (5)</td>
<td>30–40 (35)</td>
</tr>
<tr>
<td>60</td>
<td>0–&lt;1d</td>
<td>0–5 (2.5)</td>
</tr>
</tbody>
</table>

*In the AS tests seven volumes of RBC were added to one volume of sensitized RBC which had been heated at 51–60 °C.

b The NA activity was 50 % inhibited when three to four volumes separated from seven volumes of normal RBC. No separation indicated 100 % inactivation.

c The HA activity was 50 % inhibited when one to four volumes of normal RBC were agglutinated with one volume of sensitized RBC. No agglutination indicated 100 % inactivation.

d The number of minutes for which the sensitized RBC were heated before adding normal RBC. The number in brackets is the mean of the minutes required to attain 50 or 100 % inactivation.

e These values with the associated inhibition of the release of altered NDV and HN spikes from sensitized RBC appear in tables II and III. In other experiments at 56 °C the NA was 50 % inhibited between 0 and 5 min and the HA between 20 and 30 min; at 53 °C the NA was 50 % inhibited between 45 and 60 min. If inactivation is initially conducted at low temperatures such as 51–53 °C then elevated to higher temperatures the time may not accurately correspond to values from tests where the rise to the desired high temperature was rapid.

f The 50 % inactivation of the HA occurred during the rise to 60 °C.
3.3. Heat inactivation of the HA and NA activities in suspensions of NDV

The HA units declined from 64 to 4 between 32 and 60 min of 56 °C heating which increased the HI titre from 1/16 to 1/128. Thus, the mean time for 50% inactivation was 46 min. An average of two experiments (1-1.5 h and 1-2 h) gave a mean of 83 min for the 100% inactivation of the HA (Table II).

The rate of elution from the RBC decreased 50% between 9 and 12 min of heating when measured following 1.5 h of 37 °C incubation. Thus, the mean time was 10.5 min. Heating for 30 min showed a 75% decrease in the rate of elution when mea-
Table III. The comparisons of the mean times for 50 and 100% heat inactivations at 56 °C of the neuraminidase (NA) and haemagglutinin (HA) of Newcastle disease virus (NDV) estimated in tests using sensitized red blood cells (RBC) or suspensions of NDV.

<table>
<thead>
<tr>
<th>Tests</th>
<th>NA</th>
<th>HA</th>
<th>Ratios of times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 %</td>
<td>100 %</td>
<td>50 %</td>
</tr>
<tr>
<td>Sensitized RBC</td>
<td>1.5a</td>
<td>4.5</td>
<td>18</td>
</tr>
<tr>
<td>Suspension of NDV</td>
<td>10.5</td>
<td>&gt;30</td>
<td>46</td>
</tr>
<tr>
<td>Fetuin cleavage</td>
<td>78b</td>
<td>180b</td>
<td></td>
</tr>
<tr>
<td>Suspension of NDV/sensitized RBC</td>
<td>7</td>
<td>6.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Fetuin cleavage/sensitized RBC</td>
<td>52</td>
<td>&gt;40</td>
<td></td>
</tr>
<tr>
<td>Fetuin cleavage/suspension of NDV</td>
<td>7.4</td>
<td>+/-6c</td>
<td></td>
</tr>
</tbody>
</table>

a Min of heating. See Table II, footnotes c and c for the mean times of inactivation of the NA and HA at 56 °C in the agglutination—separation test.

b See Figure I for the 78 min and >180 min estimates of 50 and 100% inactivations of the NA with fetuin as the substrate. Rather than an estimate of an extrapolated value, >180 min was used in the comparisons with the other tests.

c Both values, >180 and >30, were not directly determined. Therefore the ratio is designated +/-.

3.4. Inhibition of the NA activity in suspensions of NDV estimated by the cleavage of fetuin

The amount of sialic released in tests with unheated NDV resulted in an OD of 0.70. A 50% decline to an OD of 0.35 occurred within 78 min at 56 °C; somewhat less time was required to attain a 50% decline in preparations with fetuin which were incubated for 70 min (Figure 1).

The comparisons of the NA activities in other tests versus fetuin cleavage appear in Table III.
4. DISCUSSION

The AS test estimates the effects of heat on the release of altered NDV and HN spikes from sensitized RBC, the sequential inactivations of the NA, then the HA in direct assays. Invariably the NA is more heat labile than the HA, which is consistent with the results from EI, HA and HA-1 tests [14].

The complexities of assaying the NA activity by the cleavage of artificial substrates makes the comparison of this basic reference test [2, 25] with the AS test of practical importance. At 50% inhibition the AS test was 52-fold more efficient than cleavage of fetuin (table III). In another experiment the mean time at 56 °C was 2.5 min (0–5 min); thus, the efficiency increase was 31.2-fold. The increased efficiency also occurred in the inhibition of influenza virus NA with EI antibodies [13] when compared with heat. Although heat and antibodies have different mechanisms of inhibition the increase was 8.3- to 13.2-fold greater when compared with the cleavage of fetuin [3].

Comparisons of anti-NA antibody titres show that sensitized RBC in the AS reactions versus EI tests with NDV suspensions were 12-fold and versus cleavage of fetuin were 31.3-fold greater. When compared with HI tests the titres with sensitized RBC were 4- to 8-fold greater (unpublished results). The greater anti-NA titres may be referable to fewer NA than HA determinants on the HN spikes [14]. As contrasted with NDV particles the large surface area of sensitized RBC may contribute to the ease of differentiating the HA from the NA [18, 29]; also in estimating the selective effects of heat as well as anti-NA or anti-HA antibody in polyclonal rabbit sera drawn 17 days post-immunization.

The inactivations of the NA and HA of strain 575 were sequential rather than coupled. With other strains and methods the inactivation of the HA and infectivity [23] as well as the HA and NA were coupled [24]. What appears to be coupled in the AS reactions is the separation of agglomerates and the release of altered NDV and HN spikes although the coupling is incomplete. With additional heating the separation occurs without the release. Perhaps the NA is inactivated in two phases. Another two-phase heat inactivation involving the NA has been described: first the EI antibody determinant and then a continual decrease in the rate of elution from RBC was seen [14].

The HA and NA domains [22] probably overlap to a certain extent [20, 31]. Irrespective of this overlap, heat inactivates the NA while the HA remains functional. Heat resistance implies a rigid structure [27]. Comparatively, the heat-labile NA would be less rigid than the HA. Elution may occur as a result of the flexible NA cleaving certain sialic acid–HA bonds. The NA is held in place by the rigid HA, which retains the NA configuration [7, 10].

In contrast to the mean time of 46 min for 50% inactivation of the HA in suspensions, which indicates heat stability when compared with the HA of other strains, the HA of the B1 vaccine strain is heat labile [16, 30]. The HA of B1 may be less rigid and B1 does not sensitize RBC (unpublished results). Thus, sensitization with strain 575 may result from the failure of the NA to cleave certain HA–sialic acid bonds because the HA is relatively heat resistant and rigid. Sensitization is likely to be directly related to the NA conformation.

A progressive conformational change of the NA, then the HA may be the mechanism through which heat acts. At a lower temperature, additional evidence for conformational change is that the NA on sensitized RBC is not active, whereas the NA functions at low and high temperatures if NDV has not eluted from the RBC. The sensitization process is incomplete and limitations of the NA are not imposed (unpublished results). Other structural and temperature-dependent changes have been described [17].

As shown elsewhere [16], biological purifications indicate that heat stability of the HA is a genetically stable characteristic. Heterogeneity [32] and the overall sponta-
neous mutation rate of up to 1–2% for NDV [6] should not greatly affect the AS reactions. In addition to heat stability of the HA, the other characteristics retained in the biological purifications were the ability to sensitize RBC, a relatively slow elution rate for the intact strain 575 but not its HN spikes, the responding to EI antibody, the activity of the NA on sensitized RBC requiring 37 °C temperatures, and the components of the AS reactions [14] (unpublished results). These characteristics are all referable to the conserved HA and NA domains contained in amino acid residues 171–205 [8, 19].

The heat-labile site(s) may become apparent with the three-dimensional structure of the NA, which may account for the possible two phases of the heat inactivation. The atomic structure of the NA of NDV, when known [8, 21], should allow an accurate interpretation of the effects of heat. At present, the genetically stable characteristics provide functional markers for the HA and NA domains. Residue 193 influences the HA, residues around 175 are important to the integrity of the NA of the Australian-Victorian (AV) strain [19].

The amino acid differences in the sequence near or at the NA site will help explain sensitization. The heat labilities of the NA and HA and their respective labile antibody determinants [14] (unpublished results) will have amino acid differences and perhaps configurations. The B1 vaccine strain is non-sensitizing (unpublished results), rapidly elutes [4, 30], is heat labile [30], and the HN spike has a difference of three amino acid residues when compared with the frequently studied, sensitizing AV strain [1, 4, 31]. Sensitizing or non-sensitizing strains and the effects of heat on the AS reactions soon will be understood at the amino acid and, ultimately, the atomic structure levels.

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