Title page

Antibody of undetermined specificity: frequency, laboratory features and natural history

Short running title: ANTIBODY OF UNDETERMINED SPECIFICITY

Abstract (249 words)

BACKGROUND

In pretransfusion testing at our institution, unexplained reactions are reported as antibody of undetermined specificity (AUS) after antibodies against FDA specified red cell antigens have been ruled out. The frequency, laboratory features and natural history of these reactions are not well defined.

STUDY DESIGN AND METHODS

We retrospectively examined AUS reported at a single institution between 7/1/2009 and 12/31/2011. For AUS reported in the first quarter of 2012, the reference workup and antibodies identified during subsequent testing were reviewed to characterize the laboratory features and natural history of AUS.

RESULTS

A total of 8121 antibodies were detected in 6058 patients during the study period. AUS was reported 1442 times (18%) and was the single most reported event, followed by anti-E (18%) and anti-K (14%). In the first quarter of 2012, AUS were reported in 174 unique patients. Most AUS (78%) reacted with 2 cells or less tested in gel and most reactions (98%) were 1+ or weaker. Forty-five patients presenting with AUS for the first time had repeated antibody workup later. AUS persisted in 31 cases for 2-60 days. AUS disappeared in 14 cases, 7 of which developed a total of 10 new antibodies (3 anti-E, 1 anti-D, 1 anti-C, 2 anti-Jk^b, and 1 each of anti-Le^a, anti-s, and warm autoantibody) in 3-21 days (median 8 days).

CONCLUSION

AUS is a common finding with our pretransfusion testing method. These reactions are heterogeneous and may represent antibodies against low-prevalence antigens, non-RBC antigens, or developing antibodies which are clinically significant.

KEY WORDS

Antibody of undetermined specificity, alloantibody, antibody to low-prevalence antigen, pretransfusion testing

Text (2766 words)

INTRODUCTION

A pivotal component of the pretransfusion testing is to detect and identify clinically significant antibodies in the patient samples so as to provide compatible blood. While hemagglutination and antiglobulin test remains the foundation for antibody workup,[1] the technique used in the clinical laboratory has gone through major changes over the years. The advances include the use of enhancing reagents such as low-ionic-strength saline (LISS) and polyethylene glycol (PEG),[2,3] and the development of solid-phase assay and gel microtubes.[4,5] The latter two techniques have demonstrated comparable or superior sensitivity to tube methods with LISS or PEG.[6-11] They also provide the desired platforms for automation and standardization.[12] According to recent surveys conducted by the College of American Pathologists (CAP), gel based methods has gained wide use in over 64% of laboratories in North America for antibody detection.[13]

The sensitivity of an antibody detection method may come with the caveat of detecting "unwanted" antibodies of little clinical significance.[12] These "unwanted" findings rarely provide additional benefit; instead, they may significantly increase time and effort of workup and delay urgent transfusions. Although no single method is likely to avoid detection of all "unwanted" antibodies, some studies reported detection of fewer insignificant or questionable antibodies by gel method,[8,9] while one study showed increased detection of nonspecific antibodies with a solid-phase technique.[10]

Antibody of undetermined specificity (AUS) is a term used at our institution to report unexplained reactions when antibodies against Food and Drug Administration (FDA) specified red cell antigens[14] have been ruled out based on nonreactivity with reagent red cells. This is not a universally used term, but it is consistent with the current understanding that "unwanted" antibodies may be detected during pretransfusion testing. After ruling in and out antibodies with known impact on transfusion, the

specificities of “unwanted” antibodies do not need to be determined in most cases. It is important to monitor the prevalence of AUS, because detection of excessive amount of AUS may suggest inefficiency of the testing method in use. AUS is also a relatively broad term encompassing a heterogeneous group of antibodies and it is unclear how these antibodies evolve over time. The goal of this retrospective study was to examine the frequency, laboratory features and natural history of AUS detected in the patient population of a large tertiary medical center.

MATERIALS AND METHODS

Study design and data collection

This retrospectively study examined the frequency of AUS reported at a single institution between 7/1/2009 and 12/31/2011 in relation to confirmed alloantibodies, autoantibodies and passive anti-D. Data were extracted from an electronic laboratory information system and duplicate antibodies detected in the same patients were excluded from analysis.

In the second part of the study, laboratory features of AUS reported in the first quarter of 2012 were reviewed together with the subsequent antibody identifications if available. The following parameters were collected: gender, age, type and screen result, antibodies concurrent with AUS, number of cells tested that were antigen-negative for concurrent antibodies, number of above cells that reacted with AUS, median strength of reaction, results of backup testing using tube-PEG or tube-LISS methods, reactivity of autologous control, result of direct antiglobulin test (DAT) if applicable, previous antibody history, whether AUS persisted or disappeared with new antibodies identified in subsequent workups. In cases where AUS seemed to evolve into specific alloantibodies, transfusion history and reactivity of cells with the corresponding antigens were analyzed.

Pretransfusion testing

The ID-Micro Typing System (ID-MTS) Gel Test (Ortho-Clinical Diagnostics, Raritan NJ) was used throughout the study period for antibody screen and identification. The screen was performed using a two-cell panel by the ProVue automated system (Ortho-Clinical Diagnostics) or manually. The screen cells must express the following required antigens: D, C, E, c, e, M, N, S, s, P1, Le^a, Le^b, K, k, Fy^a, Fy^b, Jk^a, and Jk^b.[14,15] Briefly, 25 μl of plasma drawn within 3 days was incubated with 50 ml of each 0.8% screen cells in the upper chamber of gel column at 37°C for 15 min followed by centrifuge at 870-920 RPM for 10 min. The gel card was inspected and graded as negative, weak, 1+, 2+, 3+ or 4+ based on standard guideline. Samples positive at screen (weak to 4+) were further tested for antibody identification. If there was no history of previous antibodies, a minimum of 8-11 cells were tested using gel card as described above. If antibodies were identified in the past, "mini" panels including 4-5 antigen-negative cells can be used to rule out antibodies of other specificities. Anti-D, C, E, c, e, k, M, N, S, s, P1, Le^a, Le^b, Fy^a, Fy^b, Jk^a, and Jk^b were ruled out using cells homozygous for the antigen or 3 heterozygous cells. Anti-K can be ruled out on one heterozygous cell. Each new antibody was confirmed in at least 3 antigen positive cells that were antigen-negative for other concurrent antibodies. With an initial impression of AUS based on gel method, additional testing was frequently performed using different panel cells by tube-PEG method (Immucor, Norcross GA). Briefly, 2 drops of patient plasma and 1 drop of reagent red cells were mixed and examined after immediate spin for hemolysis and agglutination. The mixture was then brought to the antiglobulin (AHG) phase and read macroscopically. Positive autologous control found in gel was investigated by direct antiglobulin test (DAT) using polyspecific antibodies followed by anti-IgG and anti-C3 if indicated. When DAT was positive, eluate from patient red cells was tested using a trio panel with tube-PEG method.

Statistical analysis

SPSS Statistics Version 20 (IBM, Armonk, NY) was used for statistical analysis and data visualization. Proportions were compared using Chi-Square Test. Nonparametric parameters such as percentage of positive reactions were compared using Mann-Whitney U Test. Alpha was set at 0.05.

RESULTS

Frequency of AUS detected during pretransfusion testing

During the 30-month study period, 8121 antibodies were detected in 6058 patients among a total of 138150 patients who underwent pretransfusion testing in our institute. This gave rise to an annual incidence of 2% for overall antibody detection in our patient population. AUS was reported 1442 times (18%) and specific alloantibodies 5813 times (72%). Autoantibodies and passive anti-D each comprised 5% of the total (Figure 1A). The 10 most frequently identified alloantibodies were listed in Table 1. AUS was the single most reported event, followed by anti-E (18%) and anti-K (14%). The frequency of AUS fluctuated slightly over time, being detected in 144 ± 42 unique patients per quarter (Figure 2A).

Laboratory features of AUS

In the first quarter of 2012, AUS was reported at least once in 174 unique patients with a female to male ratio of 2:1 and a mean age of 55 years (range 19-96 years). The pattern of ABO and Rh grouping was similar to the reported prevalence in the general population of United States (Table 2). No previous antibodies were identified in majority (64%) of this cohort, and AUS was the only antibody detected initially in 132 cases (76%). Forty-two patients (24%) had 1-4 concurrent antibodies, and the 3 most common concurrent antibody specificities were E, K and warm autoantibody present in 15, 9 and 7 cases respectively.

A total of 158 cases underwent gel panel testing after the initial positive screen. The reactivity of each AUS can be profiled by the number of cells tested that were antigen-negative for concurrent antibodies

and the number of positive reactions attributable to that AUS, and the accumulative count of different profiles was plotted in Figure 2A. Most samples were tested against more than 10 cells and the number of samples that reacted with 0, 1 and 2 cells were 67 (42%), 38 (24%), and 19 (12%), respectively. The median strengths of the reactions attributable to AUS were weak or 1+ in 138 of the 158 cases (97%, Figure 2B). Some AUS appeared to be highly reactive in gel, but in these cases all possible alloantibodies had been ruled out based on nonreactive cells in gel or backup testing with tube- PEG method. Figure 2C showed a subset of 17 samples that reacted with 50% or more of the cells tested in gel. When tested subsequently using tube-PEG method, 3 samples showed Rouleaux formation at immediate spin and, together with 7 additional samples, they did not react with any cells at AHG phase. Six samples reacted weakly with a range of 1 out 11 cells to 6 out of 15 cells with PEG, which was much fewer than when tested in gel. Only one sample remained highly reactive in PEG (Figure 2C, asterisk), but subsequent test using LISS-tube method was completely negative.

The autologous control (AC) was positive in 54 of the 174 cases (31%), and DAT was subsequently performed on 49 samples. DAT was positive on 29 (54%), 27 (50%) and 3 (6%) samples using polyspecific antibody, anti-IgG and anti-C3 respectively. Eluate testing was performed in 27 cases, and 8 samples reacted with all cells tested suggesting warm autoantibodies. AUS concurrent with a positive AC reacted with significantly higher percentage cells in gel than AUS concurrent with a negative AC (median 10% versus 5%, $p<0.05$), although the medium strength of reaction (≤1+ or >1+) was similar between the two groups ($p=0.26$).

Natural history of AUS

Forty-five patients presented with AUS for the first time during the study period and had at least one repeated workup later. AUS persisted in 31 cases (69%) for 2-60 days (median 8 days). AUS disappeared

in 14 cases (31%), 7 of which developed a total of 10 new antibodies (3 anti-E, 1 anti-D, 1 anti-C, 2 anti-Jk^b, and 1 each of anti-Le^a, anti-s, and warm autoantibody) in 3-21 days (median 8 days).

Figure 3 illustrated the serological changes from AUS to the 9 alloantibodies in 6 cases in relation to transfused red blood cells (RBCs). Time of the initial detection of AUS was set as zero, and the timing and units of RBCs transfused were shown as vertical bars within 30 days before and after day zero. Cases 1, 4 and 5 were transfused during the intervals of serological workups with 1 massive transfusion episode in case 5. Cases 1 and 4 also received RBCs prior to detection of AUS. The other 3 patients had no record of transfusion in our institution but could have been immunized from other sources. At the detection of AUS, those alloantibodies that were identified later were convincingly ruled out based on adequate number of nonreactive homozygous and heterozygous cells with both gel and tube-PEG methods. However, the 6 samples each reacted with 1-4 cells that express antigens corresponding to the alloantibodies identified later. The reaction strengths were weak in most cases with rare reactions up to 2+. At the disappearance of AUS, new alloantibodies were ruled in based on positive reactions with 3-8 cells at strengths ranging from weak up to 3+ and 4+. Some serological changes appeared to be incremental or graduated in nature as exemplified by cases 1 through 4, while a more potent induction of alloantibodies were observed in case 5 and 6.

DISCUSSION

The detection and reporting of AUS is by definition dependent on ruling out all known clinically significant specificities covered by FDA approved panel red cells. The residual risk associated with such unexplained reactivity is uncertain but likely to be small. Since a full crossmatch is performed in patients with AUS, most donor-recipient incompatibilities should be detected.

With our current pretransfusion testing method, which uses gel based testing at the AHG phase, the frequency of AUS was relatively high, comprising 18% of all antibodies detected. This correlates with the

general perception that gel based method is one of the most sensitive approach to antibody detection.[7,12,16] However, it is unclear whether the frequent detection of AUS is the consequence of maximizing the sensitivity in detecting clinically significant antibodies. While the RBC alloantibody specificity and frequency in different populations have been studied extensively,[17-23] the incidence of AUS has not been reported on a hospital-based patient population. This observational study presents such data from a large tertiary institution in an urban area with a racial makeup of 49% African American and 44% White. The institution provides comprehensive services to adult patients including trauma, cancer, cardiothoracic surgery, transplantation, orthopedics, high-risk obstetrics, etc., all supported by one transfusion service. The pretransfusion testing method and routine practice remained unchanged throughout the study period, and the high volume of testing allowed us to work with a relatively large sample. As more data on AUS become available from other sources in the future, the comparison of reported incidences of AUS in relation to different testing methods and patient populations would provide insight on balancing the detection of "wanted" and "unwanted" antibodies.

Since AUS remains an interpretation of exclusion, it presents a dilemma to determine what those "undetermined specificities" really are. The laboratory features of AUS described in this study may provide some clues to the heterogeneity of the entity. Many AUS we reported reacted with a single reagent cell and might have met the definition of antibody to low-prevalence antigen (ATLPA) by others.[15] However, we are among those laboratories that do not attempt to identify ATLPAs because they are often clinically insignificant and necessary reagents are often unavailable.[24,25] We also used the broader term AUS because these likely ATLPAs were not identified or confirmed. On the other end of the spectrum, some AUS were highly reactive with most cells tested in gel but the reactivity went away completely or partially with tube-PEG method. The possible mechanisms underlying these cases include non-RBC blood group antibodies,[26] including antibodies to chemicals added to RBC-suspending media, commercial antisera, or enhancement media (e.g. LISS additives in the gel column). In addition,

abnormal serum protein concentrations can produce Rouleaux formation of reagent cells in vitro.[15] A small number of cells exhibited characteristics between the two extremes, which could be due to presence of more than one unidentified ATLPAs or a combination of other mechanisms (e.g. anti-HLA antibodies). The samples with positive AC reacted with more cells in gel perhaps due to presence of low levels of autoantibodies at least in some cases that caused nonspecific agglutination of additional panel cells.

Vast majority of the AUS reacted at strengths of weak or 1+ and possible explanations include low titer or affinity of the antibodies, and preference of the antibody to react at colder temperatures. It is possible that the inclusion of "weak" when grading the reaction in our institution may introduce ambiguity where at least some reactions should have been graded as zero rather than something between zero and 1+. However, "weak" reactions were thoroughly sought for and treated similarly as 1+ reactions per guideline of the manufacturer.

The induction of alloantibodies after transfusion of red cells follows different kinetics depending on the antigen specificity but can be as short as within 3 days for E, Jk^a and K.[27] While most of the AUS (69%) persisted for 2-60 days upon repeat testing, we found 7 cases (16%) with rapid disappearance of AUS, and 6 of them developed alloantibodies against a variety of specificities (E, Jk^b, D, C, s, Le^a) with E being the most common. It is well understood that during the adaptive immune response against infectious agents, antibody producing B cells undergo V-region somatic hypermutation to improve affinity for antigen and class switch to allow distinct effector functions.[28,29] However, the experimental evidences for the affinity maturation and isotype switch in RBC alloimmunization remain sparse in the literature. Based on the pattern of serological changes in the 6 cases with newly acquired alloantibodies, there were partial reactions between the initial AUS and antigens causing the alloimmunization. The repeat workups within 3 weeks while confirming the specificities of these new alloantibodies, showed various

strength of reaction with all or most of cells tested. The greatest induction in case 4 correlated with a massive transfusion episode, which was consistent with the theory of dose effect in RBC alloimmunization.[30] Despite the lack of definitive proof, it is biologically plausible that these newly confirmed antibodies had gone through a process of affinity maturation with the initial antibodies reacting only weakly and inconsistently with cells bearing the cognate antigens. These initial antibodies may be identified as AUS, but fortunately the policy of testing only samples from within 3 days should catch and confirm any fully developed antibody later on. Thus, while most AUS may be clinically insignificant, a small fraction of AUS can serve as harbinger of future alloantibodies.

In conclusion, AUS is a common finding with our pretransfusion testing platform. These reactions were highly heterogeneous and may represent antibodies against low frequency antigens, non-RBC antigens, or antibodies in developing that may evolve into clinically significant antibodies within a short time.

TABLES

Table 1. The 10 most frequent alloantibodies detected among a hospital-based patient population in ___ (name of city omitted per requirement) during the 30-month study period (July, 2009 – December, 2011)

Alloantibody	Number of alloantibodies identified	Percentage of all alloantibodies (%)*	Percentage of all antibodies (%)†
E	1433	25	18
K	1160	20	14
D	704	12	9
Fy[a]	409	7	5
C	406	7	5
Jk[a]	297	5	4
M	282	5	3
Le[a]	249	4	3
c	195	3	2
S	127	2	1

* Total number of alloantibodies: 5813.

† Total number of all antibodies: 8121.

Table 2. ABO and Rh grouping of patients with AUS

ABO and Rh Group	Prevalence in patients with AUS* (N=173)	Prevalence in US Population (%)†	
		European Ethnicity	African Ethnicity
O	91 (53)	45	49
A	52 (30)	40	27
B	27 (16)	11	20
AB	3 (2)	4	4
Rh positive	148 (86)	84	92
Rh negative	25 (15)	16	8

* Data expressed as number of cases (%).

† Based on Ref. 15, AABB Technical Manual, 17th edition.

FIGURE LEGENDS

Figure 1. Relative frequencies of alloantibody, antibody of undetermined specificity (AUS), autoantibody and passive anti-D detected in the pretransfusion testing during the 30-month study period (A) and their quarterly changes overtime (B).

Figure 2. Laboratory features of AUS. (A) Cumulative count of AUS with different profiles of reactivity tested with various numbers of panel cells in gel. (B) Histogram of medium strength of reaction between AUS and panel cells tested in gel. (C) Change in percentage of reactivity in a subset of samples tested with gel method and tube-PEG method. Sample marked with asterisk (*) was further tested with tube-LISS method and was nonreactive.

Figure 3. Serological profiles of alloantibodies possibly developed from initial AUS in relation to RBC transfusions. Six different cases are shown. Arrows point at the timings of serological workups. See text for details.

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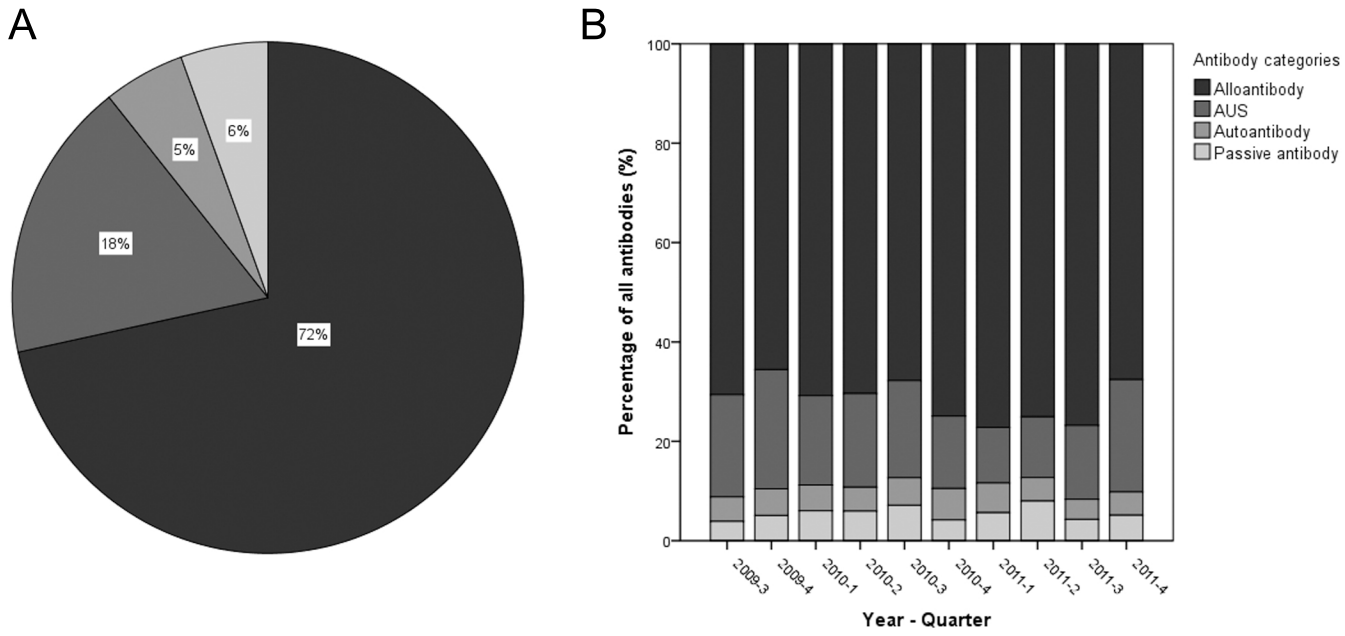
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Antibody categories
Alloantibody
AUS
Autoantibody
Passive antibody

Figure 1

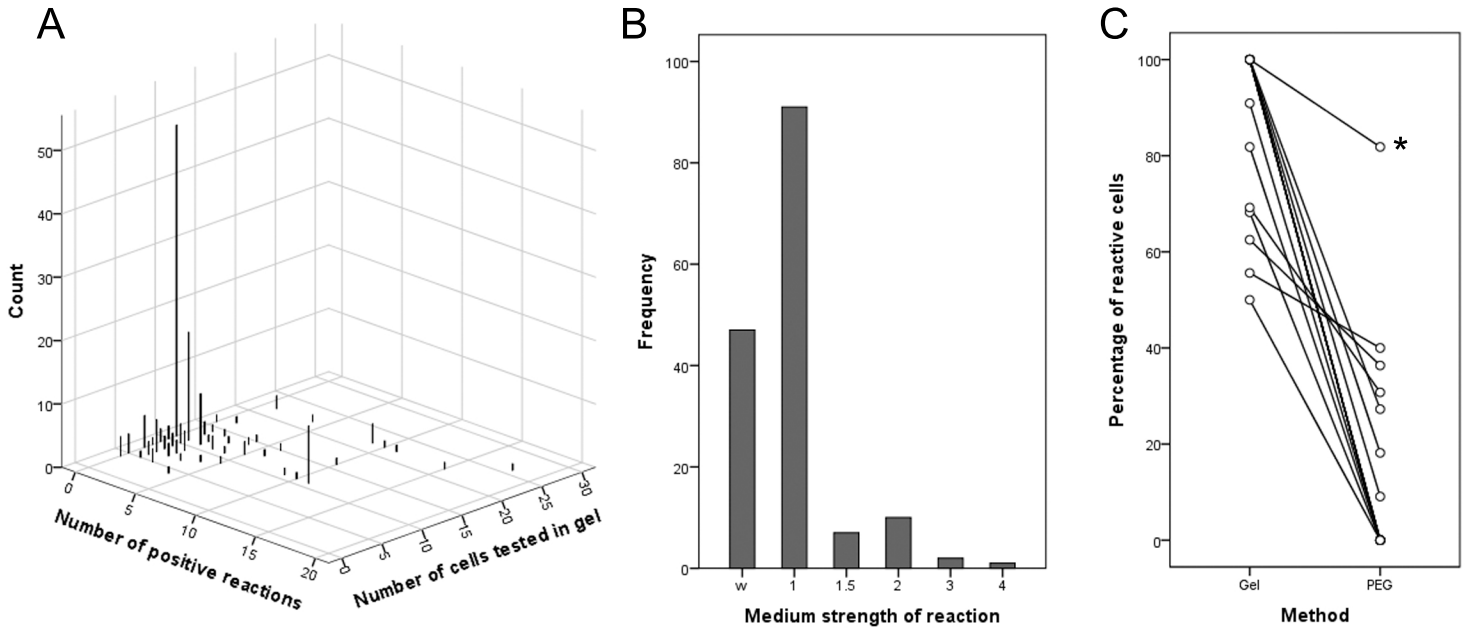
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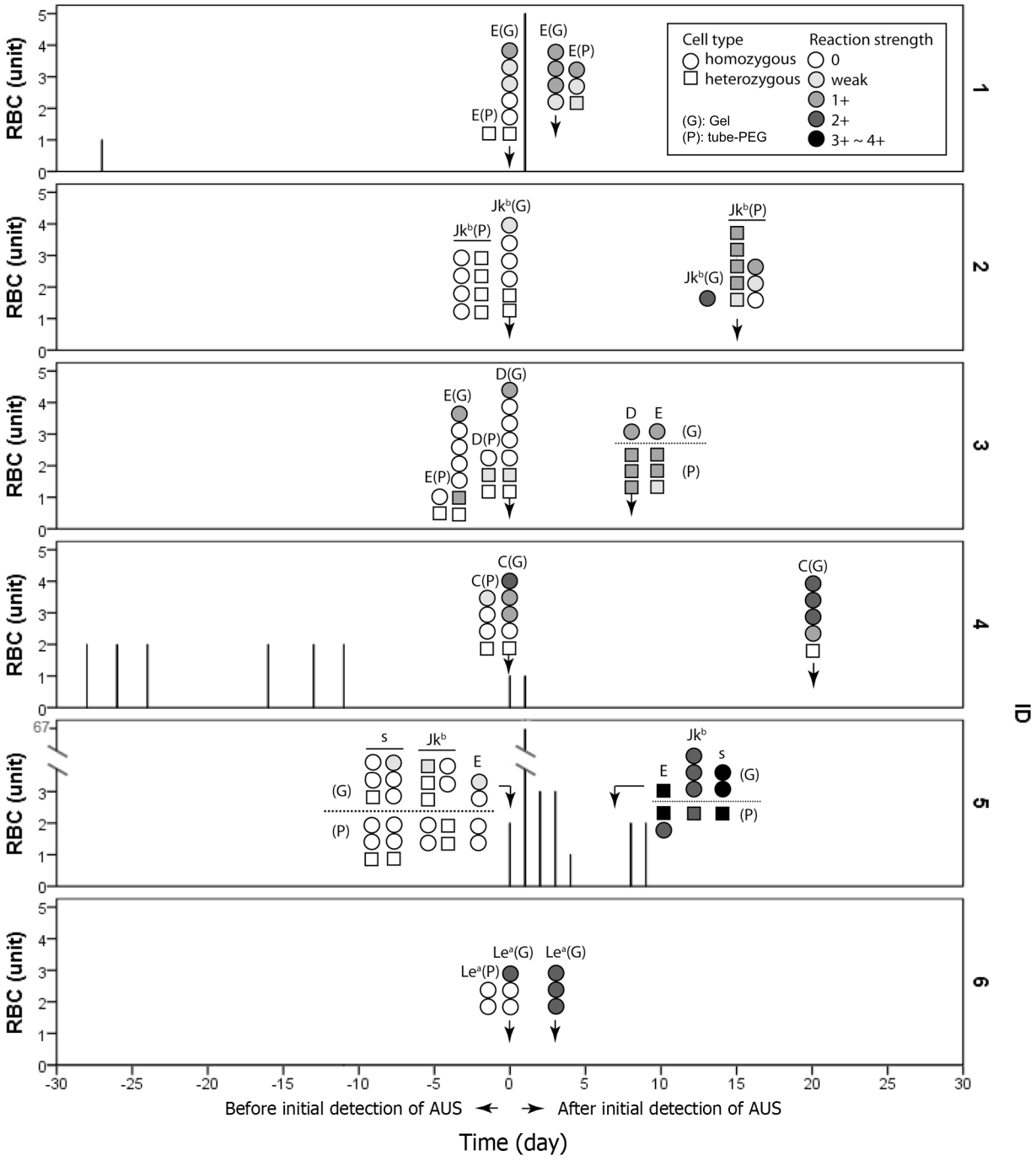
Figure 2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Le^a(P)
Before initial detection of AUS
After initial detection of AUS
Time (day)

Figure 3